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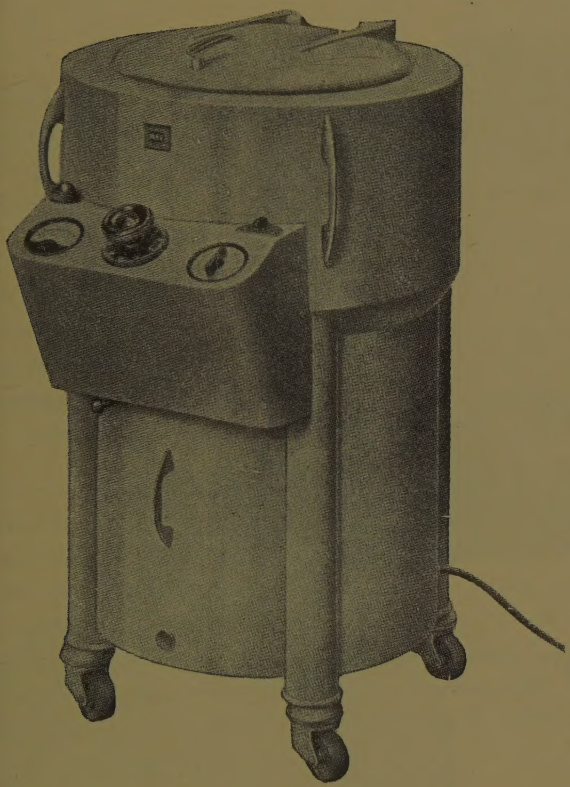

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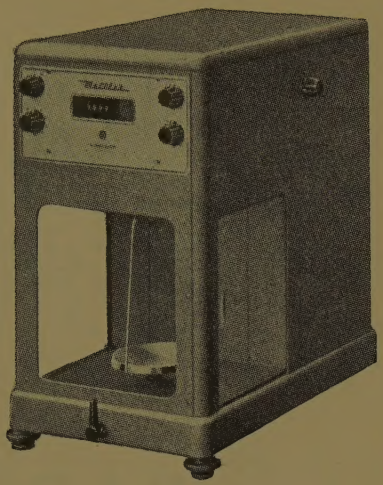

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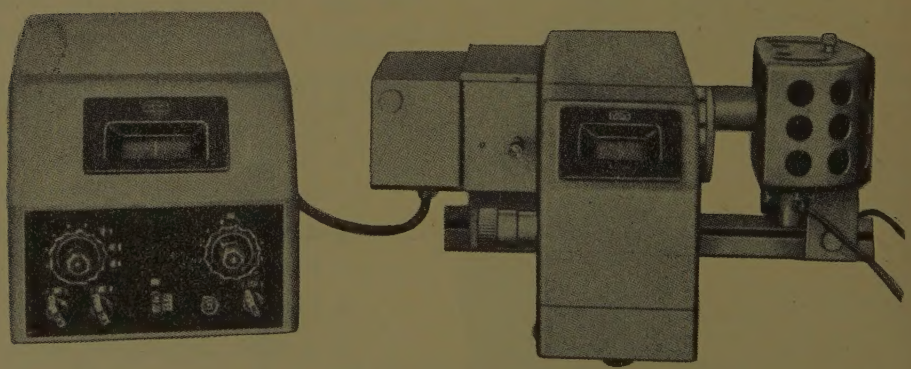

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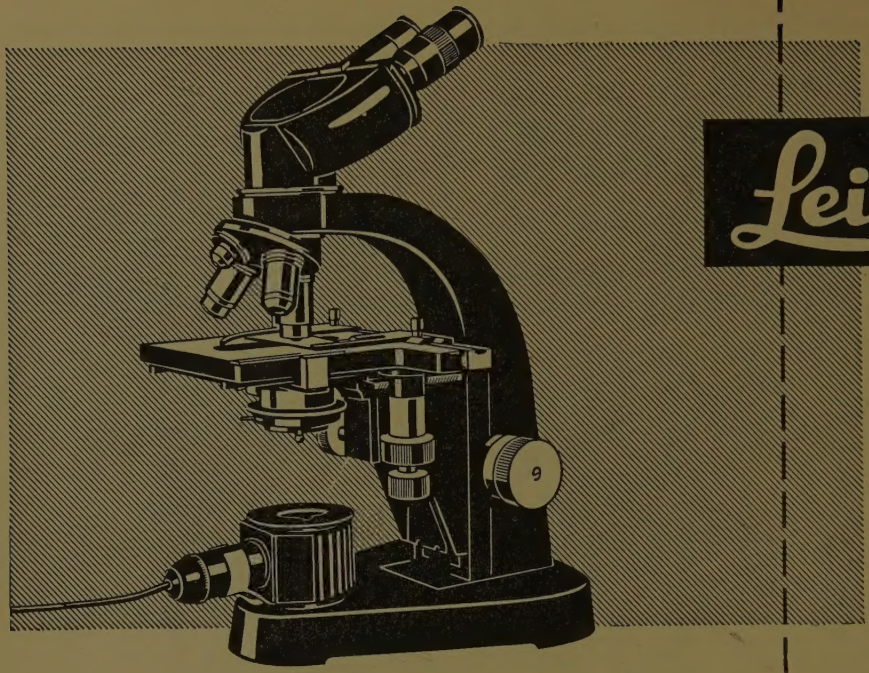

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RENAL ELIMINATION OF PENICILLIN BY THE AGED

By E. ØRNSHOLT MATHIESEN and C. TROLLE-LASSEN

rom "De Gamles By" (Old Peoples Town) and the Laboratory of Danmarks Apotekerforening, Copenhagen, Denmark

(Received for publication August 1, 1956)

A previous publication (Lind & Trolle-ssen, 1955) reported a higher and more olonged serum-penicillin concentration in ed subjects than in young ones after oral ministration of the same doses of benzyl nicillin.

It has been shown in several studies that renal clearance of a number of substances ed for renal function tests (for example, ulin, diodrast, and p-aminohippuric acid), reduced as a function of the age of the ojects (Davies & Shock, 1950; Shock, 45 and 1946; McDonald, Solomon & ock, 1951).

Consequently it was natural to assume that higher concentration of serum penicillin and in aged subjects was due to a decrease the function of the kidneys. In this con-ction it must, however, be admitted that it particularly difficult to distinguish be-een functional decreases determined by age of the subject, and those due to one more of the illnesses from which old ople often suffer.

The purpose of the present work was to investigate whether and to what degree the ability of the kidneys to excrete penicillin decreases with the increasing age of subjects. The investigations are based on a determination of the inulin clearance of the subjects, which is deemed to be the best indication of glomerular filtration, and also on a simultaneous determination of their penicillin clearance.

MATERIAL AND METHODS

Twenty subjects (two males and eighteen females) aged between 20 and 86 were used for the investigations. In consideration of the age of each individual subjects, his or her general condition was good, and none of them showed symptoms of cardiovascular or renal diseases. This does not exclude, particularly in the case of the old subjects, the possibility of the existence of such latent kidney diseases in the material as are not directly recognizable by the ordinary clinical examination (urine analysis, measurement of blood pressure, and determination of blood urea). Since the aim of the investigations in particular was to secure information about renal elimination by a random section

of the various age groups, it would have meant an undesirable screening of the material if subjects who, according to a clinical examination, generally appeared to be healthy were to be rejected after kidney-function tests because their renal capability of excretion was below some arbitrary marginal value.

In each individual instance the inulin clearance and the penicillin clearance were determined simultaneously for two periods of about 30 minutes.

75—100 ml of a 10 per cent solution of inulin was slowly injected intravenously 1—2 hours before the beginning of the first clearance period.

The penicillin preparation (2—300,000 units of a penicillin-procaine suspension) was injected intramuscularly 3—4 hours prior to the beginning of the first clearance period.

The subjects drank plenty of tepid water (about 500 ml) 2—3 hours before the beginning till the end of the periods of investigation in order to increase the diuresis and thereby to reduce the diuresis error.

The samples of blood were taken by venous punctures. A sample of blood to be used as a blank for the inulin determination was taken in advance. Moreover, samples of blood were taken at the beginning and at the end of each clearance period.

The samples of urine were obtained either by urination or by catheterization possibly followed by flushing with normal saline.

The determination of inulin in serum and urine was made colorimetrically as indicated by Bojesen (1952). The determination of penicillin in serum and urine was made microbiologically according to a method which is also used for the determination of chloramphenicol (Trolle-Lassen, 1951). The penicillin concentrations in urine were determined in suitable dilutions, while the determination of penicillin in serum was made directly. It is a well-known fact that the zones of inhibition of a given concentration of penicillin in serum are narrower than those of a corresponding aqueous solution. As the samples of serum used for this work were measured by comparison with aqueous standard solutions, a correction was necessary, and as a result of examinations of this problem the correction factor of 1.8 was used for the concentrations of penicillin found in the serum.

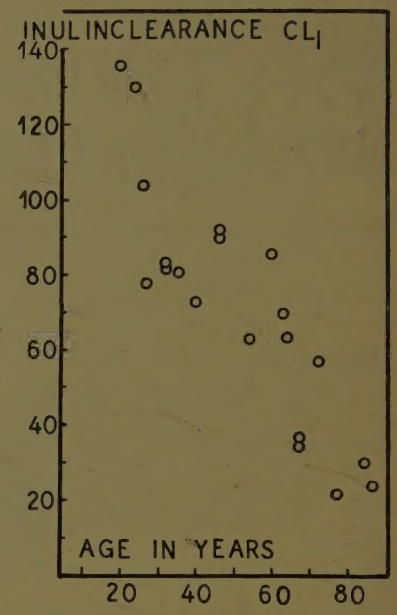


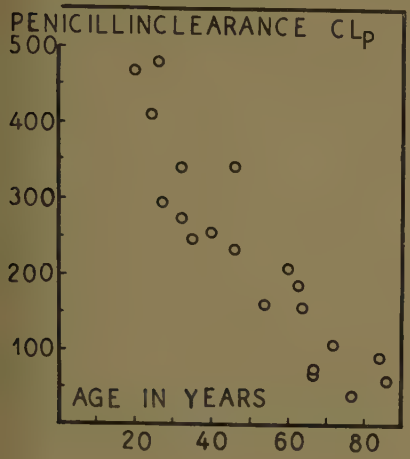
Fig. 1. Inulin clearance as a function of the a of the subjects.

RESULTS

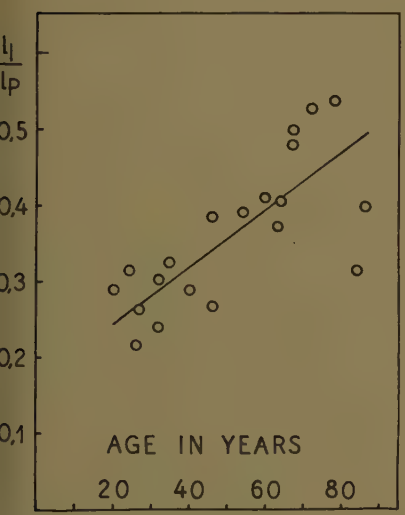
The experiments revealed a distinct variation between the age of the subject a the inulin clearance, and it will appear fr Fig. 1 that the two variables seem to inversely proportional. The inulin clearan decreases from about 130 at the age twenty to about 20 at the age of ninety.

The penicillin clearance also decreases the age of the subjects increases, and Fig seems to indicate also in this case a lin dependence between the age and the cle ance. The latter is 4—500 at the age twenty, but has dropped to 50—100 at age of ninety.

The ratio of inulin clearance to penicil clearance has been plotted in Fig. 3 a function of the age of the subject.



2. Penicillin clearance as a function of the age of the subjects.



3. The ratio of inulin clearance and penicillin rance (the filtration fraction) as a function of the age of the subjects.

rom this figure it appears that the ratio eases as age increases. In this connection ould be noted that even if the blood con- ration of aged subjects was on an aver- age a little higher than that of young subjects, the highest concentrations were only about 10 per cent of that which, according to Pers (1954), results in incipient self-depression of the tubules (25 units per millilitre). Nor was there any covariation between the blood concentration and the tubular clearance.

The straight line drawn in Fig. 3 is the regression line of the ratio of $\frac{Cl_I}{Cl_P}$ in relation to the age of the subjects. A statistical analysis of the material showed that the regression is real. If we may consider the clearance mechanism for diodrast and penicillin congruent, the fraction mentioned above $\left(\frac{Cl_I}{Cl_P}\right)$ will be an expression of the filtration fraction.

DISCUSSION

The rate of the normal glomerular filtration measured by inulin clearance is usually found to be between 70 and 200 ml per minute. The demonstrated drop in the ultrafiltration of the kidneys with increasing age (Fig. 1) has, as already mentioned, also been found by others. It is, however, far from certain that this means a reduction of the glomerular capacity of filtration. According to investigations made by Olbrich, Ferguson, Robson & Stewart (1950), it is probable that the decreasing inulin clearance is not due to a reduction in the functional capacity of the kidneys, but is dependent on factors outside the kidneys, such as changes in the vascular system dependent on age. However, the possibility of this material comprising subjects suffering from unrecognized kidney diseases cannot be ruled out.

Penicillin is eliminated through the kidneys also by tubular secretion, and presumably in a manner similar to that of diodrast with a clearance of almost the same order. Bryner, Randall & Rantz (1948) have found values of penicillin clearance between 220 and 1214 in patients who do not suffer from kidney diseases. In this country Jensen, Møller & Overgaard (1945) have found 580 in an individual subject, and Pers (1954) a normal value of 480.

Penicillin clearance, like inulin clearance, shows decreasing values with increasing age of the subjects (Fig. 2). This decrease also falls well into line with the results obtained from p-aminohippuric acid (Miller, McDonald & Shock, 1951) and diodrast (Davies & Shock, 1950), and may, to a certain degree, be traced back to the same causes as those of the decrease in the inulin clearance indicated above. However, this explanation is adequate only to the extent the relative drops in the inulin clearance and the penicillin clearance are equal.

It appears, however, from Fig. 3 that the penicillin clearance decreases at a significantly higher rate than the inulin clearance, which seems to indicate that the tubular function at any rate decreases with increasing age.

The results arrived at here show, merely quantitatively, a somewhat greater drop in the inulin clearance with age than that found by Davies & Shock (1950). These authors also found that the diodrast clearance decreased more with age than did the inulin clearance or, in other words, that there is a greater drop in the tubular function than in the glomerular function. The same observation has, to a more pronounced degree, been made in this study (Fig. 3), where the rati of $\frac{Cl_I}{Cl_P}$ (the filtration fraction) shows distinct increase with increasing age.

SUMMARY

Renal elimination of benzyl penicillin ha been investigated in humans aged betwe 20 and 90 years. The investigations we carried out by a simultaneously determin tion of renal clearance of inulin, which eliminated only by glomerular filtration, a of penicillin, which is eliminated also tubular excretion.

The investigations revealed a marked dr in inulin clearance with increasing age, co responding either to decreasing glomerul ultrafiltration or to a gradual reduction the blood flow through the kidneys.

At the same time it was demonstrated th penicillin clearance also decreases as age i creases, but at a relatively higher rate th inulin clearance.

Hence the decrease in penicillin clearan can be attributed only in part to the facto causing a decrease in inulin clearance.

The results seem to indicate that the tub lar function of the kidneys decreases w increasing age.

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A COMPARISON BETWEEN THE LÖFSTRÖM CAPSULAR SWELLING REACTION AND THE CRPA-METHOD (C-REACTIVE PROTEIN ANTISERUM) FOR DETERMINATION OF ACUTE PHAS PROTEIN IN HUMAN SERUM

By P. HEDLUND

From the Hospital for Infectious Diseases and the State Bacteriological Laboratory, Stockholm, Sweden

(Received for publication February 4, 1957)

Acute phase protein may be demonstrated in human serum with the aid of three different methods. The first was described by Tillett & Francis in 1930. They used the somatic C-polysaccharide of pneumococci as a reagent, which gave a precipitation with the active factor in serum. Löfström (1939, 1943) employed the capsular swelling reaction (CSR) of certain pneumococci. The active substance in serum caused "non-specific" capsular swelling of certain pneumococci, especially of types 27 and 28. The third method used was reported by Mac Leod & Avery (1941), Mc-Carty (1947) and Wood & Mc-Carty (1951). They isolated and purified the C-reactive protein and produced a specific antiserum by injecting the purified protein into rabbits. The acute phase protein was demonstrated by a precipitation reaction with this antiserum. As this C-reactive protein antiserum (CRPA) is commercially available, it has been possible to make a comparison regarding the sensitivity of the CRPA-method and the Löfström capsular swelling reaction (CSR).

MATERIAL AND METHODS

Sera

During November—December 1954 and October —December 1955, sera forwarded to the State Bacteriological Laboratory for determination of the acute phase protein were investigated con rently with the Löfström capsular swelling meth and the CRPA-method. Most of the patients w furnished the sera were treated in the Hospital Infectious Diseases in Stockholm.

592 sera were examined. Of these, 318 we from 145 cases of infectious hepatitis. Many these patients were bled two or more times duri the course of disease. Thus, 67 patients we represented by one sample only, while 21 patie delivered two samples, 12 patients three, 11 patie four, and 19 patients five or more samples. T rest of the material consisted of sera from 2 adult patients suffering, or suspected to suff from myocardial infarction, neoplasm, rheuma disease, nephritis or had been admitted because obscure febrile conditions. As a rule only sample was drawn from each of these 274 patien who were bled during the acute stage of disea mostly within two or three days after admissi

CSR-method

The technique used in the Löfström capsul swelling reaction is the same as that used in Ne feld's capsular swelling for typing of pneumococ The pneumococci were cultured for 18 hours hormone broth, to which 20 per cent ascites h been added. After centrifuging and washing thr times in saline, the bacteria were suspended saline containing 0.15 per cent formaldehyde. T density of bacteria in the suspension was det mined by means of Borrough and Wellcome's op city tubes. A supply of six containers with differ dead pneumococci suspensions was used. Ser twofold dilutions of pneumococci ranging from

were prepared. The strongest density, 32, corresponded to about 2,000 millions of pneumococci per ml, and density 16 to "Burrough and Wellcome's opacity tube" No. 3. The lowest density No. 1 gives 2—4 bacteria per field. One loopful of pneumococci suspension of type 27 or 23 B, one loopful of the serum to be investigated, and one drop of Löffler's alkaline methylene blue diluted 1:2 were placed on a slide by means of circular platinum loops with a fixed diameter. The three drops were mixed and covered with a coverslip. Care was always taken that the platinum loops were completely filled with pneumococci, serum and methylene blue solution, respectively, and that the three drops were mixed thoroughly, as these factors are of great importance for obtaining uniform results. After some few minutes the reaction was read in a microscope at a magnification of 1000 diameters. Every serum giving a positive reaction with pneumococci suspension No. 1 was also tested with suspensions Nos. 2—32. Serum reacting only with suspension No. 1 was said to have acute phase protein in titer 1. Serum with titer 2, 4, *etc.*, reacted with suspensions No. 2, No. 4, respectively. The use of the CSR involves two sources of error which have to be considered. The serum may contain antibodies against pneumococci, and a positive reaction can be simulated by double refraction phenomena.

However, non-specific capsular swelling substance, *i. e.* acute phase protein, causes no visible reaction in serum free from calcium ions, while the antibodies react independently of the presence of calcium ions (Löfström 1943; Abernathy & Avery, 1944). Moreover, the specific or antibody reaction is characterized by a more distinct capsular swelling than reaction. In the cases, however, where a specific capsular swelling reaction is suspected it is necessary to use the technique without calcium ions in the reaction mixture. For this purpose equal parts of a 3.8 per cent sodium citrate and Löffler's methylene blue were mixed. This solution was then used in the same way as the ordinary methylene blue solution in the CSR. When this was done, however, one drop of the serum for testing and one drop of the methylene blue solution containing sodium citrate were carefully mixed on the slide and allowed to stand for a short while before the drop of pneumococcal suspension was added. — When the results of the CSR and CRPA methods were divergent, the serum was retested with the CSR both in the presence and absence of calcium ions.

Double refraction phenomena were avoided by exact centralization of the light in the microscope.

CRPA-method

The directions for use given by the manufacturer of the CRP-antiserum (Schieffelin) have been followed except for the readings, which have been done according to another scale than that recommended. The CRPA-serum was first drawn into a capillary tube with an external diameter of about 0.8 mm. After a column of about 2 cm had been drawn up into the capillary tube, this was dipped in the patient's serum and an identical amount of serum was drawn into the tube. It was always checked that contact between the two sera was established. The capillary tube was kept in an upright position by putting one end in plasticine. The capillaries were then incubated for 2 hours at 35°C. and placed overnight in the refrigerator (4° C). The readings were made against a dark background with oblique light from above. No visible precipitation in the tube was recorded as 0 and a maximal reaction, which is seen as a heavy precipitate at the bottom of the column, as + + +. Smaller precipitates are read as + + and +.

RESULTS

The results are presented in Table I.

Table I.

CRPA	Titer	CSR							
		0	1	2	4	8	16	32	Number
	0	271	43	15	6	0	8	8	335
	+	12	24	21	8	4			69
	+ +	2	5	17	32	12	12	8	88
	+ + +	0	0	7	15	28	31	19	100
	Number	285	72	60	61	44	43	27	592

Among the 592 sera examined, no visible reaction with either of the two methods was recorded in 271. With the CSR, acute phase

protein could be demonstrated in 307 sera (72 + 60 + 61 + 44 + 43 + 27) and the CRPA method gave a positive reaction in 257 sera (69 + 88 + 100). A ++ or +++ reaction was found in 188 sera with the CRPA test, and a titer of 4 or more was found in 175 sera with the CSR test.

78 sera (13 per cent) did not give corresponding results with the two methods used. The CSR was positive in 64 sera (43 + 15 + 6; 11 per cent; titer ranging from 1 to 4), while the same sera did not react with CRPA. Conversely, in 14 sera (2 + 12; 2 per cent) which gave a + or ++ reaction with the CRPA method, no acute phase protein could be demonstrated with the CSR.

Among the 64 sera giving a positive CSR and a negative CRPA reaction were 40 from cases of infectious hepatitis and 24 from cases with various other diseases.

Only a few patients who had delivered these 64 sera were feverish during the 48 hours preceding the collecting of samples. Thus, 55 of the 64 sera in this group came from patients without fever. Eight sera came from patients with subfebrile temperatures and suffering from infectious hepatitis (5), rheumatoid arthritis (1), congestive heart failure (1), or carcinoma of the bile ducts (1). One patient with acute cystitis had a febrile temperature.

In the group of 14 patients who had delivered sera with positive CRPA reaction but negative CSR, 10 suffered from infectious hepatitis and 4 from various other diseases.

In this group only one of the patients suffering from infectious hepatitis was feverish. This patient was treated for hepa coma. Three patients treated for chron bronchitis, myocardial infarction and su pected carcinoma of the pancreas, respec vely, all had subfebrile temperatures. T remaining 10 patients were afebrile.

128 sera, which gave inconsistent resu with the two methods, were retested. In ni cases such a retesting gave discordant r sults, as shown in Table II.

Table II. *The reaction of nine se giving discordant results on (the first a second tests) retesting.*

Number of sera	First test		Retest	
	CSR titer	CRPA	CSR titer	CRPA
5	1	0	1	+
1	2	+	2	++
1	8	+	8	++
1	0	+++	2	++
1	0	++	1	++

Hence, five sera, which gave a titer 1 r action with pneumococci but a negati reaction with CRPA, on retesting show the same reaction with CSR but a + r action with CRPA. In two sera the CRP reaction was positive in the first test b negative with CSR. In these nine cases t inconsistent results are explained as due methodical error.

None of the 64 sera giving a positive CS but a negative CRPA reaction contain antibodies against pneumococci type When tested in the absence of calcium io the sera gave no visible reaction with t pneumococcus type 27 capsule.

DISCUSSION

The different methods to determine acute se protein have earlier been compared as ards their sensitivity. With the antiserum duced by Mac Leod & Avery (1941) it possible to detect smaller amounts of eactive protein in an acute phase serum n it was when the C-polysaccharide of umococcus according to Tillett & Francis 30) was used. Löfström (1944) made a nparison between the reactions of acute se serum with pneumococcus C-poly-charide and with pneumococcus type 27. confirmed that the C-polysaccharide and pneumococcus type 27 reacted with the ne substance in human acute phase prote- He had some sera which gave a positive umococcus type 27 reaction but a nega- C-polysaccharide reaction. None of his were positive to the C-polysaccharide negative to type 27. Wood & Mc Carty 51) compared the sensitivity of the speci- antiserum method with the sensitivity of reaction with C-polysaccharide. They wed that the C-polysaccharide reaction not reliable at serum C-protein concen- ions below 0.1 mg/ml, while their quanti- ve method with rabbit antiserum gave itive reactions in the presence of less n 0.01 mg/ml. These workers further ceeded in demonstrating C-reactive pro- in sera from patients with viral hepa- , as was also demonstrated by Hedlund 47). The amount of C-reactive protein he sera of Wood & McCarty, however, too low to give a visible reaction with C-polysaccharide.

n the present study the CSR seems to equally sensitive as the CRPA method in infectious hepatitis. Thus, 40 out of 564 sera were positive with CSR and negative with CRPA, while the reverse was demonstrated with 10 sera. The CSR has been used on a vast material of infectious hepatitis, and has proved very valuable in following the course of the disease (Hedlund, to be published).

On the whole, the results of this investigation showed a good conformity between the CSR and CRPA methods. So far there is nothing to suggest that one method would be more sensitive than the other in a special disease.

For routine work the CSR and CRPA methods are convenient to use. For ordinary personnel of a clinical laboratory not trained in using the microscope for bacteriological examinations, the CRPA method seems to be preferable because it is easier to read.

SUMMARY

1. The acute phase protein in 592 serum samples was investigated by means of Löfström's Capsular Swelling Reaction (CSR) and C-reactive Protein Antiserum (CRPA).

2. The two methods seem to be equal as to sensitivity.

3. For persons lacking experience in bacteriological microscopy the CRPA method is to be preferred, as it is easier to read.

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E THIOCYANATE CONTENT IN URINE AND BLOOD FROM CASES OF TOXEMIA OF PREGNANCY

By C. NYSTRÖM and BO SÖRBO

From the Department of Woman's Diseases, Karolinska Sjukhuset, and the Department of Biochemistry, Medicinska Nobelinstitutet, Stockholm, Sweden

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was recently suggested by Grauert 53, 1954) that the toxic agents in eclamp- (toxemia of pregnancy) is hydrocyanic , formed by the fetus. Hydrocyanic acid etoxified in the body by conversion to cyanate (Lang, 1933), and in support of theory Grauert claimed to have demon- ted an increase of thiocyanate excretion ases of eclampsia. The analytical method l by Grauert must, however, be consi- d to be rather unspecific, and we have sequently tried to verify Grauert's results a more specific and sensitive method the determination of thiocyanate. As s of fully developed eclampsia seldom ır in Sweden, some determinations were e on samples from the initial stages of mia. If Grauert's theory is valid, an in- se of thiocyanate content in blood and e must be expected also in these cases.

METHODS

hiocyanate was determined by a modification e method of Boxer & Richards (1952). Thio- ate is first oxidized to cyanide under mild itions in acid solution, and the hydrocyanic liberated is then aerated into alkali and deter- d colorimetrically with Zincke-König's re- n.

the present work the initial steps of Boxer Richards have been retained, but the cyanide aerated into 4 ml of 0.1 M NaOH and the colorimetric determination of the cyanide was carried out according to the more simple procedure of Asmus and Garschagen (1953). The color was thus developed by the addition of 0.8 ml 1 M NaH_2PO_4 and 0.2 ml 1 per cent chloramine-T solution, followed 2 minutes later by 1 ml of a barbituric acid-pyridine reagent (8 g barbituric acid and 16 ml pyridine diluted with water to 50 ml). The optical density at 580 mμ was then determined 7—15 minutes after the addition of the last reagent and the thiocyanate content in the sample obtained from a standard graph.

RESULTS

Thiocyanate was first determined in blood and urine from cases of normal pregnancy

Table I. *Thiocynate content in blood and urine from cases of normal pregnancy.*

Case no.	Blood serum CNS^- μg/ml	Urine CNS^- μg/ml	CNS^- excretion mg/24 h
4417—54	1.04	1.55	2.48
4467—54	1.82	2.18	-
4978—54	6.00	2.35	3.28
5182—54	1.55	1.80	2.69
4769—54	1.93	2.08	1.04
3958—54	-	3.95	2.37
1129—55	0.82	0.78	1.33
0874—55	5.58	2.52	6.02
1024—55	0.42	1.50	0.75
1084—55	1.81	2.11	2.95
1068—55	0.99	1.12	1.30
Mean:	2.20	2.19	2.42
Range:	0.42—6.0	0.78—3.95	0.75—6.02

Table II. *Thiocyanate content in blood and urine from cases of toxemia.*

Case No.	Diagnosis	Blood Serum CNS^- µg/ml	Urine CNS^- µg/ml	CNS^- excretion mg/24 h
4828—54	Toxemia	2.28	4.00	5.60
M.E.—55	Toxemia	1.80	1.37	1.64
V.G.—55	Toxemia	2.27	1.27	-
E.R.—55	Toxemia	2.91	5.06	-
4409—54	Preeclampsia	1.66	1.57	1.10
1144—55	Preeclampsia	-	0.68	0.64
3969—54	Eclampsia	-	3.51	3.51

(Table I). When these results are compared with those obtained on samples from cases of toxemia (Table II), it is immediately evident that no significant difference exists between the groups and no relation between toxemia of pregnancy and the thiocyanate content of urine and blood serum could be demonstrated. Grauert's theory for the genesis of eclampsia thus appears to be untenable.

DISCUSSION

From Table I it is evident that large variations occur in the thiocyanate content of blood and urine from cases of normal pregnancy. The thiocyanate present in the body is believed to be derived in part from cyanide forming glucosides and mustard oil glucosides taken up with the food, in part from preformed thiocyanate in the food and, finally, from cyanide, endogeneously formed in the body (Stuber & Lang, 1934). Since the thiocyanate content in the body fluids is related to different constituents in the food, large variations can be expected to occur in patients not kept on a very strict diet. Another factor, not considered in the present work, is tobacco smoking, which has been shown to increase the thiocyanate conte of blood and urine (Lawton, Sweency Dudley, 1943; Maliszewski & Bass, 195 Our values for the thiocyanate content blood are in the same range as those fou by other workers with similar metho (Boxer & Richards; Maliszewski & Bas and those obtained by Stuber & Lang (193 with a different colorimetric method. O values for the urinary thiocyanate are in t same range as those reported for norm cases by Bauman *et al.* (1934), Lawton *et* and Maliszewski & Bass. Grauert, howev reported that in cases of eclampsia the th cyanate in a 24 hour urine sample was creased from the normal value of 30—50 to 150—350 mg. His normal value is m than 10-fold higher than the values found other workers. Grauert determined the u nary thiocyanate by an iodometric titrati according to Rupp & Schied (1902), but was pointed out by Bauman *et al.* that t method sometimes gives up to 20-fold high results when applied to urine samp The error was attributed to the presence certain reducing sulfur compounds in urine.

SUMMARY

he thiocyanate content of urine and se- samples from cases of toxemia has been rmined and compared with that obtained n cases of normal pregnancy. No increase the thiocyanate content was found in emia.

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DETERMINATION OF PANCREATIC LIPASE IN HUMAN SMALL INTESTINAL CONTENT

By B. BORGSTRÖM

From Swedish Medical Research Council, Unit for Metabolic Studies University of Lund, Lund, Sweden

(Received for publication March 5, 1957)

We have earlier described a method for the assay of pancreatic lipase in pancreatic juice and gland extracts (Borgström, 1956). This method is based on the rate of clearing of an artificial triolein emulsion, the rate of clearing being proportional to the amount of lipase added. Determination of pancreatic lipase in small intestinal content is, however, complicated by the presence therein of bile constituents. For the assay of lipase in small intestinal contents the method therefore has to be modified so that the effect of the bile constituents is eliminated. This can be done in two ways. The assay can be performed at low pH values (4—6) in the presence of a large excess of added bile acids, or the bile constituents of the intestinal content can be separated from the proteins by precipitation of the latter with ethanol in cold. Of these two alternatives the last one was found the simplest and most reliable and will be described here.

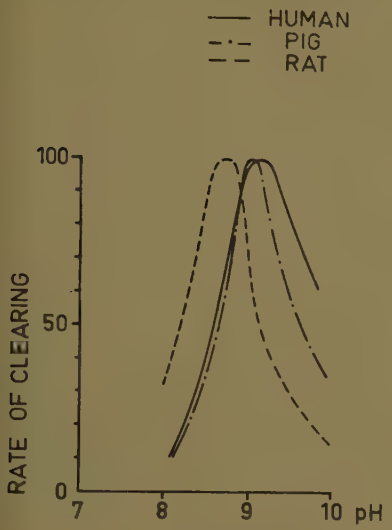
EXPERIMENTAL

Substrate

1 g of triolein (Merck), 100 mg of 1-mono-olein (Destillation Products) and 100 mg of oleic acid are dissolved in 50 ml of acetone. 2 ml of this solution are slowly stirred into 48 ml of 0.05-m Tris buffer pH 9.1. In this way a stable and reproducible emulsion is obtained. Two parts of this emulsion when diluted with one part of the sa buffer should give an optical density of approx mately 1.0 when measured in a one cm cell 650 mμ. The emulsion is prepared fresh each da

Determination of lipase activity

The intestinal content is collected in flasks chill on ice. 0.5—1.0 ml thereof is transferred as soo as possible to 15 ml centrifuge tubes containir 2 mg trypsin-inhibitor dissolved in 0.5 ml of buff pH 8.0. 10 ml of ethanol (at —10° or lower) a added to the centrifuge tube and the tube allow to stay in ice for half an hour. After centrifugati for 5 minutes the supernatant is discarded and t precipitate suspended in 10 ml cold ethanol a centrifuged again. The precipitate is then di solved in 9.5 ml of 0.05 m Tris buffer pH 9.1 room temperature and immediately assayed. F the assay 2 ml of the above emulsion are put in 1 cm cell; 1 ml or less of the enzyme solution added, the final volume always being adjusted 3 ml with buffer. The rate of clearing of the emu sion is measured in a spectrophotometer at 650 m by determining the time for the decrease in optic density from 0.90 to 0.80. This time is inverse proportional to the amount of lipase added. T amount of lipase added should, however, be a justed so that the time of clearing from 0.90 to 0. is between 1 and 5 minutes. One lipase unit is giv as the amount of lipase that gives a decrease optical density from 0.90 to 0.80 in 1 minute. T lipase unit has been selected so that small intestir content from human beings during digestion giv values around 50—100 units per ml. The dete minations have been performed at room temperatu but the use of equipment for keeping constant te perature during the measurements is advisable



1. pH-rate of clearing relationship for human, t and hog pancreatic lipase using a triolein emulsion as substrate.

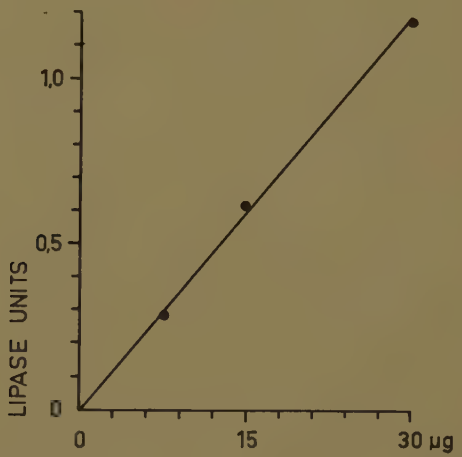


Fig. 2. Relationship between lipase units and micrograms of human pancreatic juice protein assayed according to the method described.

EFFECT OF pH ON THE CLEARING REACTION

ig. 1 shows the pH-activity relationship he clearing reaction. The pH optimum human pancreatic lipase [1] with the subte described above was found to be 9.1—compared to 8.7 for rat pancreatic lipase 9.0—9.1 for hog pancreatic gland lipase. 2 shows the lipase-rate of clearing reonship with human pancreatic juice pro- at pH 9.1. From Fig. 3 it is apparent no straight line relationship exists when ll intestinal content is assayed directly. however, the proteins are precipitated with ethanol in the same sample of intestinal content, a straight line relationship is seen. Thus it appears that the intestinal content contains substances which inhibit the rate of clearing at pH 9.1. These substances are removed by the ethanol precipitation. From other experimental data it seems fair to assume that this inhibition is an effect of bile constituents (Borgström, 1956).

Experiments in which human pancreatic juice protein has been added to human small intestinal [2] content and the lipase activity assayed according to the method, show that the recovery of lipase is practically complete in the precipitation procedure (Fig. 4). Re-

The human pancreatic juice was collected from nts, the pancreatic ducts of whom were can- ed for therapeutic purposes by Dr. Doubilet ellevue Hospital, New York. The juice was ediately lyophylised and stored as dry powder hich form the lipase is stable.

[2] The small intestinal content was collected by intubation from the upper jejunum of a healthy human after a test meal containing milk protein, glucose and corn oil.

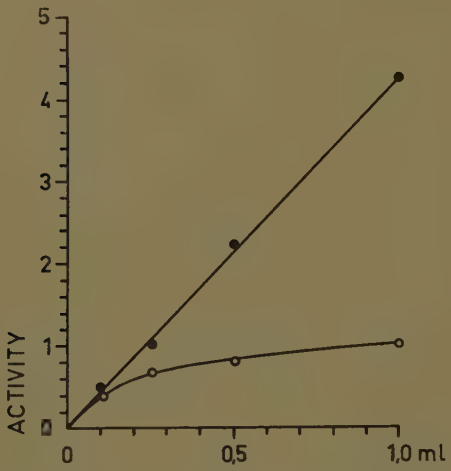


Fig. 3. Effect of increasing amount of human small intestinal content on rate of clearing (o — o). Same after precipitation with ethanol (● — ●).

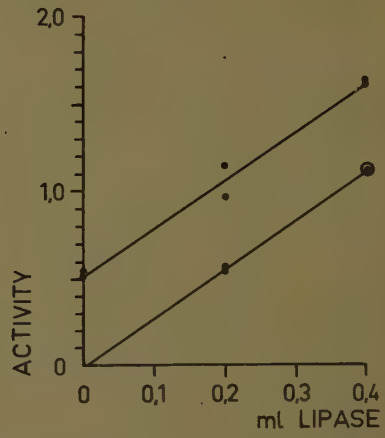


Fig. 4. Recovery of human pancreatic juice lip added to human small intestinal content. Lo curve: human pancreatic lipase assayed direct Upper curve: human pancreatic lipase added human small intestinal content and assayed acco ing to the method described.

peated washings with ethanol, however, result in a gradual decrease in lipase activity.

The lipase in small intestinal content is rapidly inactivated on storage. At 0° the activity falls to about 50 per cent in 24 hours. Addition of trypsin inhibitor partly prevents this inactivation. Twelve different samples of human small intestinal content were assayed immediately after collection and after storage at 0° with added trypsin inhibitor (crude lima bean inhibitor (Whortington)). The mean activities remaining after 1 and 3 days were 88.5 and 81.5 per cent. It is therefore important to assay for lipase in intestinal content as soon as possible after collection.

SUMMARY

A simple method for the determination pancreatic lipase in human small intesti content, is described. The method is bas on the rate of clearing of an artificial tri lein emulsion after precipitation of the pr teins of the intestinal content with etha in cold.

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DETERMINATION OF TRYPSIN AND CHYMOTRYPSIN IN HUMAN INTESTINAL CONTENT

By G. LUNDH

m The Swedish Medical Research Council, Unit for Metabolic Studies, University of Lund, Sweden

(Received for publication March 5, 1957)

earlier determinations of proteolytic vity in pancreatic and duodenal juice, hods have been used by which only the proteolytic activity was assessed, for mple Anson's method with hemoglobin stion. In recent years, however, specific trates have been introduced (see Neu- & Schwert, 1950) and with these it is ible to determine the amount of trypsin chymotrypsin in the same sample. De- inations of the enzymatic activity in *in- nal juice* with specific substrates have erto, however, been reported only by mond & Schwachmann (1955). They rmined chymotrypsin in duodenal con- , making use of benzoyl-phenyl-alanine- thyl ester as substrate.

his paper is concerned with a method the parallel determination of trypsin and notrypsin in human intestinal content. s method is a modification of Schwert Takenaka's (1955) spectrophotometric hod for determination of these enzymes ure solutions.

MATERIALS

-tyrosine (TYR) and tyrosine ethyl ester ochloride (TEE) from Eastman Kodak were in the chymotrypsin determinations. In the in determinations were used benzoyl-arginine) and N-benzoyl-arginine ethyl ester (BAEE), synthetized according to Bergmann, Fruton & Pollok (1939), Schwert, Neurath, Kaufman & Snoke (1948), and Schwert & Eisenberg (1949).

Standard reference curves were made, using crystalline chymotrypsin from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A., crystalline trypsin with 50 per cent $MgSO_4$, also from Worthington, and finally crystalline trypsin, "Trypure", from NOVO Terapeutisk Laboratorium, Copenhagen, Denmark.

For the determination of trypsin 0.05 M tris-buffer, pH 8.0 with 0.02 M $CaCl_2$ was used, and for the determination of chymotrypsin the same buffer with $CaCl_2$ but with pH 7.0.

METHODS

The spectrophotometric determinations were made in a Unicam spectrophotometer with 1 cm quartz cells.

For the determination of chymotrypsin 0.002 M solutions of TYR and TEE in tris-buffer pH 7.0 were used. The difference in optical density between the TYR and TEE solutions was first checked with 2 ml solution and 1 ml buffer in the cuvettes. The determinations were made at a wavelength of 233.5 mμ and a slit width of 1.5 mm. The difference in optical density between TYR and TEE must be .6—.7 in transmittance. Then the spectrophotometer was adjusted so as to give an optical density reading of zero with 2 ml TYR and that amount of the test solution which was to be analyzed, and the final volume adjusted to 3 ml with buffer. Next, 2 ml of the TEE-solution was placed in the other cuvette, the test cuvette, with the same amount of the test-solution and buffer as in the standard cuvette. The speed with which the dif-

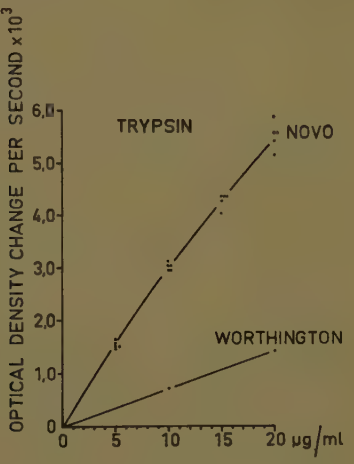


Fig. 1. Reference curve for trypsin.

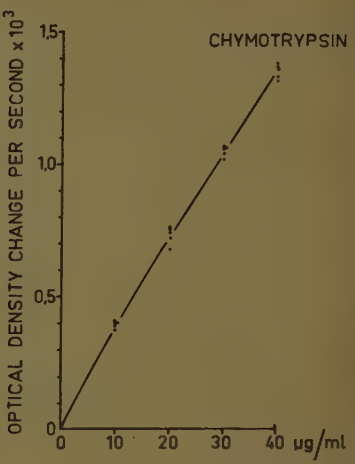


Fig. 2. Reference curve for chymotrypsin.

ference in optical density was reduced was measured. The time for the decrease in optical density from .4—.3 in the test cuvette was measured.

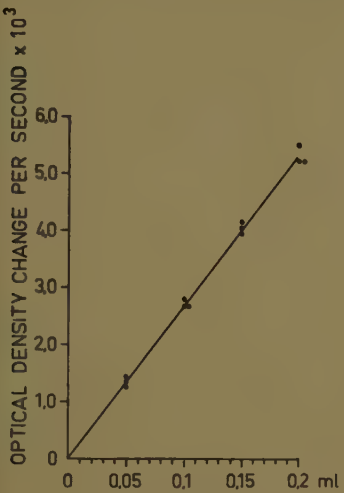
In determining trypsin the method was modified since BA is more light-absorbing than BAEE (Schwert & Takenaka, 1955). Here 0.001 M solutions of BA and BAEE in tris-buffer pH 8.0 with 0.02 M $CaCl_2$ were used. The spectrophotometer was adjusted so as to give an optical density reading of zero with 2 ml BAEE and 1 ml of the buffer. The determinations were made at a wavelength of 253 mμ and a slit width of 1.5 mm. The difference in absorption of the BA and BAEE solutions (2 ml of the solution and 1 ml of the buffer in each cuvette) was first measured and must be .5—.6 in transmittence. Two cuvettes were then filled, one with 2 ml of BA solution and the amount of the test solution to be analyzed, the other with 2 ml of BAEE solution and the same amount of the test solution as in the BA cuvette. Both were then adjusted to a final volume of 3 ml with buffer. With the BAEE test cuvette in the light path, the time for an increase in the optical density from .2 to .3 was measured. If, however, the test solution is light absorbing, one has to make allowance for this when measuring the speed of hydrolysis. If, for example, the difference between BA standard cuvette and BA test cuvette in light absorption was .2 in transmittance, the time for an increase of optical density in the BAEE test cuvette fr .4 to .5 was measured.

All the determinations have been made at ro temperature, but to exclude the influence of chan in room temperature it is recommended to equipment for making the determinations at a c stant temperature.

When standard solutions of trypsin and chy trypsin were made, the optical density was de mined at 280 mμ and 282 mμ respectively and concentrations calculated according to Kunitz (19 and Schwert & Kaufman (1951).

The enzymatic activity was given in mi grams/ml, referring to standard reference cu with known amounts of enzymes. It is, howe difficult to obtain pure enzyme preparations the standard curves. Only one preparation chymotrypsin (Worthington) has been avail and the standard curve for this is seen in Fig Standard curves for trypsin were made both Worthington's trypsin and with Trypure NOVO, and there is a significant difference tween these two preparations, as is seen in Fig In the following, Trypure NOVO is used as try standard.

Richmond & Schwachmann (1955), when de mining chymotrypsin in duodenal content, trypsin inhibitor since benzoyl-phenyl-alanine m thyl ester is also to a certain degree hydrolyze



3. Tryptic activity with various amounts of test solution in the cuvettes. Trypsin has been ed to a dissolved precipitate of inactivated intestinal juice to maintain 100 μg/ml.

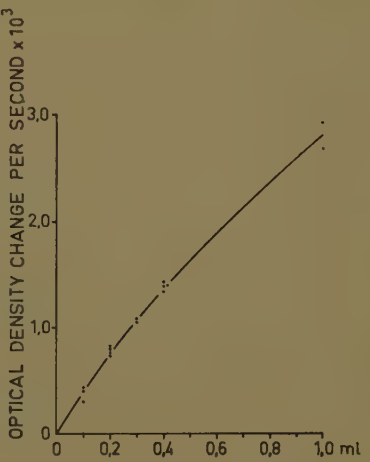


Fig. 4. Chymotryptic activity with various amounts of the test solution in the cuvettes. Chymotrypsin has been added to a dissolved precipitate of inactivated intestinal juice to maintain 100 μg/ml.

sin (Ravin, Bernstein & Seligman, 1954). With micrograms of chymotrypsin in the cuvettes e is only a very slight hydrolysis of BAEE, esponding to 1 microgram/ml of trypsin. TEE ot hydrolyzed by trypsin, not even with 100 rograms of trypsin in the cuvettes.

DETERMINATION OF ENZYMES IN INTESTINAL JUICE

When measuring the enzymatic activity in stinal contents spectrophotometrically, greatest difficulty is the presence of sub- ces which absorbs light at the wavelength d for the trypsin and the chymotrypsin erminations. This is why only very small ntities can be analyzed and why the de- ninations are time-consuming and im- ctical. In order to eliminate the main part these substances, especially degradation ducts of proteins, the proteins of the in- inal juice were precipitated by ethyl hol and redissolved in 0.9 per cent NaCl tion.

One ml of intestinal juice is pipetted into centrifuge tubes with 9 ml of cold, absolute alcohol (—15° C). Then the tubes are placed in crushed ice for about 30 minutes and centrifuged. The supernatant is decanted and the precipitate dissolved in 0.9 per cent NaCl solution. Sometimes the precipitate was not completely dissolved and the solution had to be centrifuged before the spectrophotometric determination was made.

The procedure of alcoholic precipitation renders the spectrophotometric determinations of the enzymes more accurate, especially as regards the chymotrypsin. Without alcoholic precipitation one can only make the determinations with 0.01—0.05 ml of intestinal juice in the cuvettes; after alcoholic precipitation one is able to work with 0.05—0.1 ml.

The reference curves are obtained with pure enzyme solutions. In order to show that

various concentrations of the dissolved alcoholic precipitate from intestinal content in cuvettes do not influence the determinations, the following experiment was carried out. Trypsin and chymotrypsin were added to dissolved precipitate of intestinal juice which had been kept at room temperature for some days and which consequently had lost all enzymatic activity. The solution was made so as to contain 100 micrograms of trypsin and chymotrypsin per ml, and the determinations were made with varying amounts of this solution in the cuvettes. The curves from these determinations were almost identical with the reference curves. (Figs. 3 and 4.)

Comparison between enzymatic activity in intestinal juice before and after alcoholic precipitation shows that no reduction in activity is brought about by the precipitation procedure. Ten determinations of trypsin were made from the same sample of intestinal juice before and after alcoholic precipitation. The mean value before precipitation was 160.5 micrograms per ml ($\pm$ 2.1) and after 169.0 micrograms per ml ($\pm$ 4.0). The same procedure for chymotrypsin yielded 281.0 micrograms pr. ml ($\pm$ 5.5) before and 261.0 ($\pm$ 7.1) after the alcoholic precipitation.

The alcoholic precipitate can be stored for at least 3 days at — 15° C without decline in enzymatic activity.

Intestinal juice has been incubated at + 4° C and at + 37° C. After 7 hours there was no change in enzymatic activity at + 4° C. At + 37° C, however, there was a distinct decline in activity. Thus, intestinal juice can be collected in flasks kept in crushed ice without any reduction in enzymatic activity.

SUMMARY

With a modification of Schwert & Takenaka's (1955) method for spectrophotometric determination of trypsin and chymotrypsin with specific substrates, a method has been designed for determination of these enzymes in human intestinal content.

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TIMATION OF THE PLASMINOGEN ACTIVATOR AND THE RYPSIN INHIBITOR IN ANIMAL AND HUMAN TISSUES

By TAGE ASTRUP and O. K. ALBRECHTSEN

From the Biological Institute of the Carlsberg Foundation, Copenhagen, Denmark

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lasmin, the protease in blood, is formed n its precursor, plasminogen, by various vating agents, Astrup (1956). One of e is the plasminogen activator in tissues. content of this activator in various tis- has been studied by Permin (1947, 0) and by Lewis & Ferguson (1950), its insolubility has prevented its quanti- ve estimation. Astrup & Stage (1952) nd that the tissue activator could be e water soluble by treatment with potas- n thiocyanate. This observation has now le it possible to devise a method for the ntitative extraction and estimation of the ue activator. The stability of the tissue vator at acid reaction, Astrup & Stern- ff (1956), has permitted its separation n the labile plasminogen activator pre- in blood and has made the method ctive for the estimation of the tissue vator. The method also permits a sepa- estimation of inhibitory agents, such as trypsin inhibitor found in ox lung tissue Astrup (1952). It is the purpose of this er to describe in detail the estimation cedure, which has yielded satisfactory lts in routine use for nearly two years.

MATERIALS AND METHODS

he plasminogen activator was estimated on lard fibrin plates with 0.1 per cent fibrinogen (bovine, containing plasminogen) according to Astrup & Müllertz (1952). Bovine thrombin (kindly supplied by *Løvens Kemiske Fabrik*, Copenhagen) was used for clotting. The activities were recorded as the diameter products in sq.mm. of the lyzed zones after 20 hours at 37° C (average of three separate estimations). Dried pig heart tissue (containing the plasminogen activator) was prepared from fresh hearts (Astrup, 1951 b). From this a water soluble activator was prepared as a powder (Astrup & Sterndorff, 1956), or as described below. Tissues from pig heart, ox lung, human myometrium and organs from rat and man were used fresh or stored at —20° C. Dried ox lung tissue was used as the source of the protease inhibitor (pulmin) (Astrup, 1952).

Solutions of the trypsin inhibitor in human urine (Astrup & Sterndorff, 1955), were concentrated according to Astrup, Alkjær & Soardi (1957). The trypsin inhibitor in soy beans was prepared about 50 per cent pure after a method slightly modified from Kunitz (1946). Inhibitor activity was estimated against trypsin by means of fibrin plates heated at 85° C. for 35 minutes (Lassen, 1952). The pure crystalline trypsin ("Trypure", containing 24.4 Anson units per g) was kindly supplied by *Novo Terapeutisk Laboratorium*, Copenhagen. The stock solution contained 0.25 mg per ml in 0.025-n HCl and was diluted 2:15 in barbital buffer immediately before use. The diethyl barbiturate buffer (0.05-M, pH 7.8) contained 0.1-M NaCl. Total $\mu = 0.15$.

Streptokinase ("Varidase"), kindly supplied by the *Lederle Laboratories*, was used in the activation of the plasminogen activator in blood (Müllertz & Lassen, 1953; Müllertz, 1955).

RESULTS

1. *Influence of KSCN on the activity estimation:*

Since potassium thiocyanate is used as a solvent for the tissue activator, its influence on the activity estimation was investigated. Solutions of the water soluble tissue activator (40 mg per ml) were made in 0.9 per cent NaCl or in varying concentrations of KSCN. The results of such an experiment are presented in Fig. 1. The estimations are not significantly influenced by the presence of even high concentrations of KSCN. The KSCN alone (in various concentrations) has no lytic effect on the fibrin substrate. Deviations occurring at low activities are often observed in estimations with the plate method. These deviations are independent of the presence of KSCN. They are caused by small and varying amounts of inhibitory agents in the fibrin substrate.

2. *Extraction of the tissue activator:*

The concentration of KSCN needed for extraction of the tissue activator was estimated as follows: Pig heart powder (1000 mg) was shaken for 2 hours with 10 ml 0.9 per cent NaCl or varying concentrations of KSCN respectively.

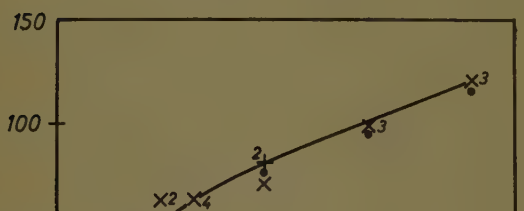


Fig. 1. Effect of KSCN on the estimation of the activity of the tissue activator. *Abscissa:* Concentration of the tissue activator in per cent of the stock solution. *Ordinate:* Activities on standard fibrin plates. The points represent the following solutions: 0.9 per cent NaCl; 0.15-M KSCN; 0.50-M KSCN; 2.0-M KSCN.

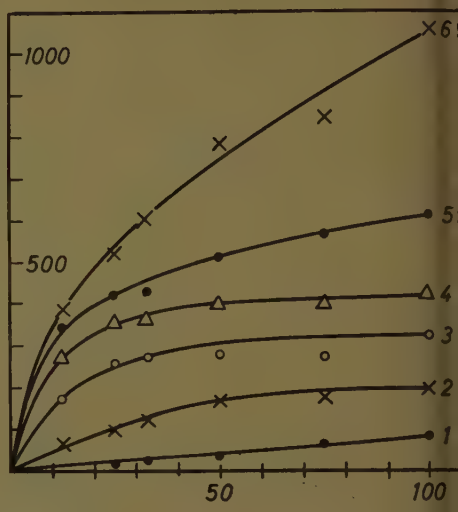


Fig. 2. Effect of the concentration of KSCN the extraction of the tissue activator. *Abscis* Concentrations in per cent of the original extra The dilutions were made with the same concent tion of KSCN as used for the extraction. *Ordina* Activities on standard fibrin plates. *Curve 1:* per cent NaCl; *Curve 2:* 0.15-M KSCN; *Curve* 0.2-M KSCN; *Curve 4:* 0.6-M KSCN; *Curve* 1.0-M KSCN; *Curve 6:* 2.0-M KSCN.

After filtration the activities in Fig. 2 recorded. Increasing activities were obtained increasing concentrations of KSCN. The solution gave a very low activity. Further ex ments showed that a 2-M solution of KSCN the optimal concentration for the extraction.

A quantitative extraction of the tissue activa was obtained after three extractions. When 1000 of fresh pig heart tissue was homogenized Potter homogenizer with 2-M KSCN and tracted repeatedly for one hour with 10 ml KSCN, the activities of the second and t extract correspond to about 20 per cent and 5 cent respectively of the first extract. The fo extract was inactive.

3. *Extraction of the protease inhibitor*

Ox lung contains large amounts of a prot inhibitor which can be isolated by a treatment potassium thiocyanate (Astrup, 1952; Astrup

, 1956). If an inhibitor of this type is present issue it is contained in the thiocyanate extract red as described above for the extraction of ssue activator. It was found that the three ctions needed for the quantitative isolation of issue activator were also sufficient for the itative extraction of the inhibitor present in ox lung tissue.

recipitation of the tissue activator

e KCSN-extract prepared as described above ins the tissue activator and possibly also a ase inhibitor of the type found in ox lung. In to estimate the activator it is therefore essen- o devise a procedure for the quantitative ation of the tissue activator from the protease tor. This can be accomplished if one takes tage of the fact that the tissue activator is itated by acidification (Astrup & Sterndorff, while the lung inhibitor will remain in the natant thiocyanate solution (Astrup & Stage, A complete separation of the tissue activa- rom the trypsin inhibitor was obtained as vs:

extract of dried pig heart with 2-M KSCN cidified with 1-N HCl. After centrifugation the sediment was redissolved to the original volume with 2-M KSCN by neutralization with solid $NaHCO_3$. Complete precipitation of the active agent was obtained at pH 1. Complete recovery was also obtained with fresh tissue from pig heart, pig brain and pig lung, but not with human myometrium, which is a very active tissue. Therefore human myometrium (2000 mg) was homogenized and treated for 2 hours with 2-M KSCN (40 ml). After centrifugation aliquots were diluted 16 times with 2-M KSCN and H_2O so that the final concentrations of KSCN varied from 0.125-M to 2.0-M. The diluted solutions were precipitated by 1-N HCl at pH 1.0 and the sediments redissolved to the volume of the original undiluted extract and compared in activity with the original untreated solution. The recoveries increased with increasing dilutions until 0.25-M KSCN, where the recovery was quantitative. Further dilution decreased the yield. After the diluted solution was acidified, precipitation occurred rapidly and complete recovery of activator activity could be obtained from the precipitate. Table I demonstrates the reproducibility of the precipitation from an extract of fresh pig heart. Human myometrium gave similar results.

Table 1. *Recovery of activator activity after acid precipitation of seven aliquots of a 0.25-M KSCN solution of pig heart. The recorded activities are the average values of triplicates for each dilution of the redissolved precipitate. The concentrations of the dilutions are expressed in per cent of the undiluted solution. The time elapsed before precipitate is redissolved has no influence.*

Conc. in per cent	Activities after:						
	0 min.	5 min.	10 min.	15 min.	20 min.	25 min.	30 min.
100	412	422	399	427	445	426	420
75	374	374	380	387	367	387	399
50	318	318	330	348	324	318	342
25	256	245	261	267	267	267	261
12.5	200	160	192	191	196	192	192
6.25	144	136	144	143	144	144	136

5. *Separation of the tissue activator from the bovine lung inhibitor*

After the conditions for a quantitative precipitation of the tissue activator had been devised a complete separation from the trypsin inhibitor present in bovine lung was accomplished. First the separation of the tissue activator from mixtures of pig heart and ox lung was studied.

2000 mg fresh pig heart tissue was homogenized in a Potter homogenizer and shaken for one hour with 30 ml 2-M KSCN and centrifuged. Similarly, 1000 mg fresh ox lung tissue was treated with 25 ml 2-M KSCN. Aliquots of the two supernatants were mixed (Solution A). A sample of A was diluted 8 times with H_2O and brought to pH 1.0 with 1-N HCl. After centrifugation the sediment was dissolved to the original volume with 2-M 2-KSCN after neutralization with $NaHCO_3$ (Solution B). The activity of B was compared with the activity of A and with the activity of the original extract from pig heart (twice diluted). The resulting dilution curves are seen in Fig. 3. The activity of the tissue extract is decreased

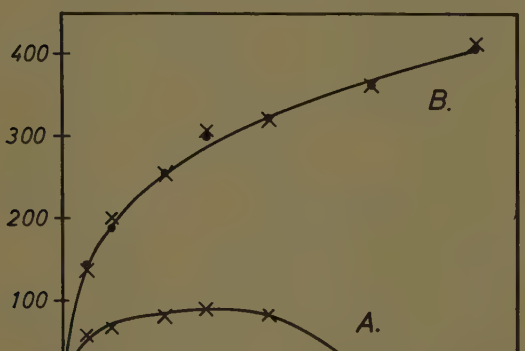


Fig. 3. Recovery of the tissue activator from a mixture with the bovine lung inhibitor. *Abscissa:* Concentration of the solutions in per cent of the undiluted solution. *Ordinate:* Activity on standard fibrin plates. Curve ●——● = activities of the original pig heart extract equally diluted; curve A = activity of the mixed extract from pig heart and ox lung; curve B = activity of the separated tissue activator.

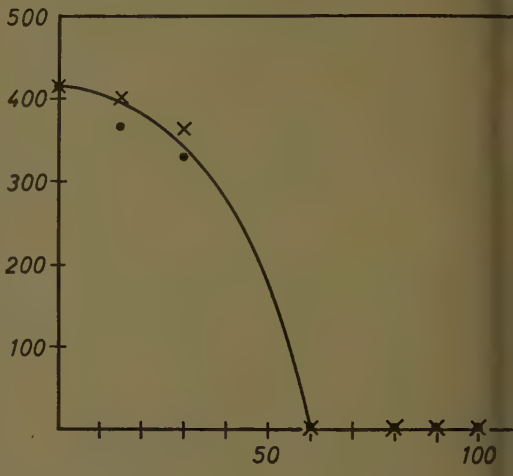
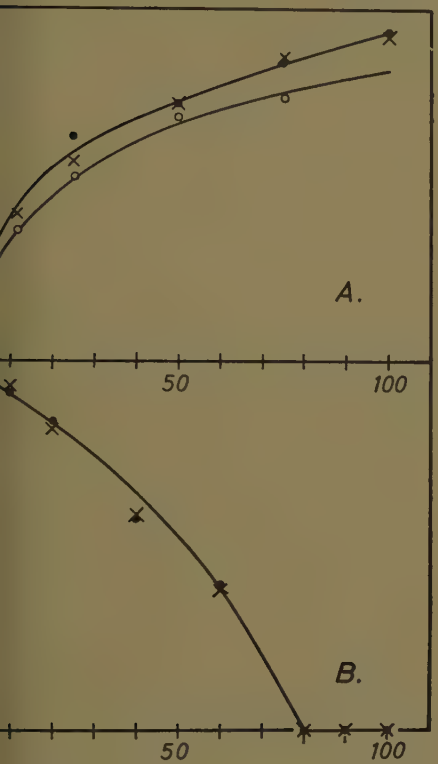


Fig. 4. Recovery of the lung inhibitor from mixture with pig heart tissue. *Abscissa:* Conc tration of the solutions in per cent of the undilu solution. *Ordinate:* Activity on heated fibrin pla Equal volumes of the diluted solutions and trypsin solution were mixed. Curve x——x activity of the original lung extract (equally luted). Curve ●——● = activity of the recov inhibitor solution.

considerably by the lung inhibitor especially at higher concentrations of the mixture. Dilu apparently favours the activity of the activ though it is not sufficient to approach a qua tative estimation. However, complete recovery the tissue activator is obtained by the acid pre tation. The lung inhibitor is retained in the su natant from the acid precipitation, and should quantitatively precipitated by addition of sod tungstate (Astrup & Stage, 1956). Therefore, 0. sodium tungstate (0.1 vol.) was added to the su natant from the acid precipitation. After 30 at room temperature the precipitate was separ by centrifugation and redissolved to the orig volume in barbital buffer. The inhibitory act of this solution was estimated against trypsin heated fibrin plates (in order to avoid the e of any contaminating activator) and compared the inhibitory effect of the original lung ti extract (equally diluted). The results are sh in Fig. 4. Apparently complete recovery of inhibitor is obtained.



5 A and B: Recovery of the tissue activator
he urine inhibitor from a mixture of these.
bscissa: Dilutions in per cent of concentra-
of the stock solution. *Ordinate:* Activity on
ard fibrin plates. Curve ●——●: Activity of
solution of tissue activator. Curve o——o:
aty of mixture with the urine inhibitor.
e x——x: Activity of tissue activator after
ation by acid precipitation. B: *Abscissa:* Di-
ıs in per cent of concentration of the stock
on. *Ordinate:* Activity against trypsin on
d fibrin plates. Curve x——x: Activity of
solution of the urine inhibitor. Curve ·——·:
ity of the inhibitor solution after precipation
e mixture with the tissue activator at acid
reaction.

separate experiments dilutions of an extract
vine lung were similary treated, and the in-
ry activities estimated and compared with a
on curve of the original extract. No signi-
ficant differences were found. Further control experiments were made with varying proportions of pig heart extract and ox lung extract. They showed that the activator concentration and inhibitor concentration could be estimated independent of the relative concentrations of activating and inhibiting agents in the mixture.

6. *Separation of the tissue activator from the urine inhibitor*

Urine contains varying amounts of a protease inhibitor which can be separated from the plasminogen activator in urine by an adsorption process, Astrup & Sterndorff (1955). The effect of this inhibitor on the estimation of the tissue activator was investigated because its presence in kidney tissue could interfere with the estimation of activator concentration in this tissue.

3000 mg fresh pig heart tissue was homogenized and treated with 10 ml 2-M KSCN. After centrifugation the stock solution was twice diluted and an activity curve was made on normal fibrin plates. One ml of a suitably concentrated solution of the urine inhibitor was diluted to 20 ml with 2-M KSCN. The inhibitory effect of this solution (twice diluted) was estimated against trypsin on heated fibrin plates. Equal parts of the undiluted solutions were then mixed, and the activity of this solution was estimated on the normal plates. The mixture was then diluted 8 times with distilled H_2O, acidified to pH 1.0, and the activator and inhibitor separated and estimated as described above. The results are presented in Fig. 5 A and B. It is seen that complete recovery of the tissue activator, as well as of the urine inhibitor, is obtained. It is also seen that the activator activity is not decreased so much in a mixture with the urine inhibitor as was the case with the lung inhibitor (see Fig. 3). This is in accordance with the results previously published, Astrup & Sterndorff (1955). Contamination with the urine inhibitor is apparently without influence on the estimation of the activator.

7. *The tissue activator and the soy bean inhibitor*

Attempts were also made to separate the tissue activator from a mixture with the trypsin inhibitor

present in soy beans. The experiments were performed in a similar manner as described above for the lung inhibitor and the urine inhibitor. The results of these experiments were not satisfactory. It was not found possible to separate quantitatively the tissue activator and the soy bean inhibitor. This may indicate a chemical difference between the soy bean inhibitor and the above mentioned inhibitory agents.

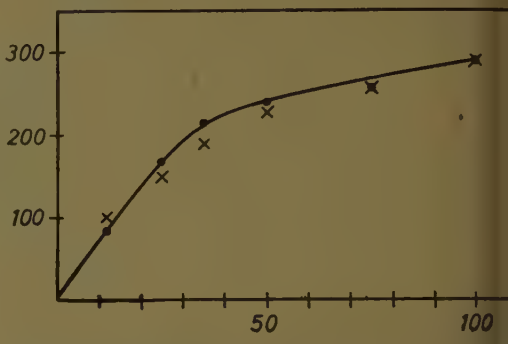


Fig. 6. Estimation of tissue activator in the p
sence of streptokinase activated human plas
Abscissa: Concentrations in per cent of the origi
solution. *Ordinate:* Activity on standard fi
plates. Curve ●——● : Dilutions of the origi
pig heart extract. Curve x——x: Dilutions of
solution recovered from a mixture with strep
kinase activated human plasma.

8. *Estimation of the tissue activator in the presence of blood plasma*

Blood contains agents inhibiting fibrinolysis (antiplasmin, antitrypsin) which belong chefly to a group of labile inhibitors. Furthermore blood also contains fibrinolytically *active* agents belonging to the group of labile plasminogen activators or to their precursors (for reference see Astrup, 1956). The transformation of the activator precursor to the activating agent can be carried out by addition of streptokinase. Similar systems are found in various fluids in the organism. All tissues which are being investigated for the presence of the tissue activator of plasminogen are contaminated with inhibitory and active substances derived from blood or other fluids of the organism. For that reason the effect of the blood system on the estimation of the tissue activator was investigated.

Fresh human plasma (0.05 ml) activated by addition of an excess of streptokinase was added to 2.5 ml of a fresh pig heart extract prepared with 2-M KSCN. The activated human plasma contains large amounts of the labile plasminogen activator and of inhibitory agents. The amount of plasma added could only be reached if more than half of the weight of the tissue investigated consisted of blood. The mixture was precipitated as described above and the activity recovered in the acid precipitate estimated on standard fibrin plates. The recorded activities were compared with those obtained directly from the original heart extract, see Fig. 6. There is very little difference between the results obtained, and contamination with even large amounts of the components in blood does not interfere significantly with the estimation of the tissue activator. As a further control the inhibitory effect against trypsin was estimated in fresh plas (on heated fibrin plates) and compared wit similar sample acidified with 1-N HCl to pH and left for some minutes before neutralizati Nearly all the trypsin inhiitor in plasma destroyed by this treatment.

9. *Control experiments with various organs*

In order to establish the applicability of method to the estimation of the tissue activator a variety of tissues, different organs were inve gated. Fresh tissue (500 mg) from human org (heart, brain, ovary, skeletal muscle) and rat org (heart, brain, lung, kidney) were homogenized extracted three times for one hour with 2 KSCN as described in Section 2. The activities the extracts from different tissues before precipitation were estimated on standard fi plates and compared with the activities recor after acid precipitation (Section 4). No signific differences were recorded indicating that the e mation method can be applied to tissues of diffe types.

Final description of the procedure:

he final method for estimating specifically the minogen activator in tissue and the trypsin in-or is then as follows:

resh tissue (1000 mg) is homogenized in a er homogenizer with 2-M KSCN and trans-d to a glass tube, shaken for one hour with a of 10.0 ml 2-M KSCN and centrifugated. The ment is again treated for one hour with 10.0 ml KSCN and this treatment is repeated once. three supernatants are combined. An aliquot is drawn and diluted with 7 parts of distilled) and acidified to pH 1.0 with 1-N HCl. After rifugation the sediment is redissolved in 2-M CN to the original volume of the aliquot and ralized on litmus paper with solid $NaHCO_3$. activity of serial dilutions of the activator tion is estimated on standard fibrin plates.

o the supernatant fluid after acidification, of its volume of 0.1-M sodium tungstate is ed. After 30 min. the sediment is separated by rifugation and dissolved to the original volume he aliquot in barbital buffer (pH 7.8; 0.05-M; aining 0.1-M NaCl). The inhibitory activity of al dilutions was estimated by mixing with equal mes of the trypsin solution and measuring the vity of heated fibrin plates.

The accuracy of the activator estimation

he standard deviation of the method was esti-ed as described by Astrup & Müllertz (1952). standard deviation of a single estimation was estimated. An activator solution was prepared n fresh pig heart by acid precipitation as de-bed above. A dilution curve of this stock solu-was obtained on the standard fibrin plates in usual way. Three different dilutions were prepared and the activity of each dilution nated on 10 fibrin plates (each with three rate estimations). For each estimation (average he triplicates) the concentration was obtained nterpolation on the dilution curve of the stock tion. The standard deviation on a single deter-ation (σ) is then

$$\sqrt{\frac{\Sigma\, d^2}{n-1}}$$

where n = number of determinations and d = differences from the mean concentration. The following results were obtained:

Mean conc. = 79; $n = 10$; $\Sigma\, d^2 = 267$; $\sigma = 5.45 \simeq 7$ per cent of the mean concentration.

Mean conc. = 60; $n = 10$; $\Sigma\, d^2 = 76$; $\sigma = 2.9 \simeq 5$ per cent of the mean concentration.

Mean conc. = 13; $n = 10$; $\Sigma\, d^2 = 2$; $\sigma = 0.47 \simeq 4$ per cent of the mean concentration.

Only dilution curves of identical slopes can be compared because different slopes indicate that the activities obtained are caused by qualitative differences in the composition of the solutions. The double logarithmic graphs make it easy to observe if the solutions can be compared in terms of concentration or if they differ qualitatively. This advantage has been stressed before, Astrup (1938, 1950). The methodical error in the estimation of the slope of the dilution curve is therefore of significance for the accuracy of the method and was estimated as follows: To ten samples (each of 2.50 ml) of a pig heart extract was added 17.50 ml distilled H_2O and 1-N HCl until pH 1.0 After centrifugation the sediment was neutralized and dissolved in 2-M KSCN to 2.50 ml and dilutions (100; 75; 50; 25; and 12.5 per cent) were prepared in 2-M KSCN.

The activities of these dilutions were estimated on normal fibrin plates (average of triplicates) and the ten dilution curves were drawn as usual on a double logarithmic graph. The slopes (a) of the curves were calculated as the increase in log c with one unit's increase on the abscissa, when the concentration (c) is expressed in percentages of the stock solution. The following results were obtained for the deviation in estimating the slope: Mean slope: 0.48; $n = 10$; $\Sigma\, d^2 = 0.0030$; $\sigma = 0.018 \simeq 4$ per cent of the mean slope.

The standard deviation of the estimation of the concentration by interpolation from a dilution curve upon a standard curve was then esimated. The values A_{100} for the activities of the 100 per cent concentration of the dilution curves were read on the standard curve and the average value $\bar{A}_{100}$ calculated. The activity equation:

$$\log A = \bar{a} \log c + \bar{b}$$

relates c (the concentration) with A (the activity

estimated) as a straight line with $\bar{a}$ and $\bar{b}$ as the parameters, and expresses thus the standard curve. Since

$$\log \bar{A}_{100} = \bar{a} \log c_{100} + \bar{b}$$

$$\bar{b} = \log \bar{A}_{100} - 2\bar{a}$$

when c_{100} is expressed in per cent. Hence

$$\log A = \bar{a} \log c + \log \bar{A}_{100} - 2\bar{a} \text{ or}$$

$$\log c = \frac{\log A - \log \bar{A}_{100}}{\bar{a}} + 2$$

On the ten dilution curves the values of A for $c = 100$ were read and the concentration c calculated. The deviation was calculated for each dilution curve and the standard deviation of the interpolated result was then calculated as follows:

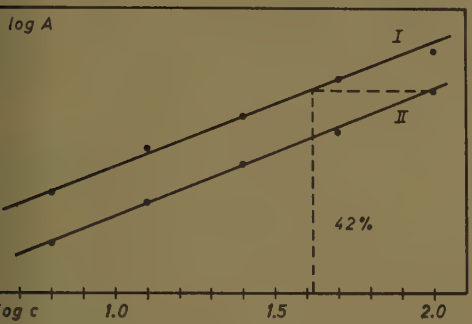
Mean concentration calculated $(\bar{c}) = 102$; $n = 10$; $\Sigma d^2 = 470$; $\sigma = 7.2 \simeq 7$ per cent of the mean concentration. This deviation includes errors in the preparation of the solutions, including the acid precipitation.

12. *The standard curve:*

Because the sensitivity of the fibrin plates varies with each new fibrinogen preparation, it is necessary in comparative investigations to use a reference curve from a standard preparation of the tissue activator for interpolation and calculation of the activities. The properties of the bovine blood from which our fibrinogen is made change slightly from one animal to another. It contains various amounts of fibrinogen and inhibitory agents so that the resulting preparations of fibrinogen deviate too much from each other to serve as a standard substrate for a direct estimation of the activities when more accurate determinations are needed. The fibrin plates, made with different batches of fibrinogen, vary in their sensitivity to the fibrinolytic agents. When the fibrinolytic activity (in sq.mm) is plotted on double logarithmic paper, curves with different slopes are obtained. It is also noticed that when serial dilutions of low fibrinolytic activity are plotted, there may be a deviation from the straight line in the higher dilutions. A suggested explanation for this phenomenon is that the effect of the inhibitory agents present in the fibrin plate plays a larger role at low conce trations of the active components. For use in comparative investigations a standard preparat of the tissue activator was therefore made follows:

Fresh pig hearts were treated and dehydrat with acetone as described by Astrup (1952). T tissue powder was treated for 2 hours with 2- KSCN (100 ml per 10 g). After centrifugation supernatants was diluted 8 times with distill water and 1-N HCl added to pH 1.0. The sedim was separated by centrifugation and redissolved the original volume in barbital buffer. Insolu matter was removed by centrifugation. The soluti was lyophilized and stored in closed bottles —20° C. The preparation was apparently sta when stored in the dry state at —20° C. It has b in use for nearly two years as a standard p paration and other preparations have been m and compared with it for use as substandar Care has to be taken against any contaminati with water vapors. The preparation is hygrosco and deteriorates rapidly when moist. At one ti one of our preparations lost one third of its a vity during a few weeks because it had not b kept fre from water vapor. It must be placed i desiccator during equilibration of the temperat from —20° C to room temperature before A solution containing 30 mg per ml of the stand preparation (dissolved in 2-M KSCN) produ considerable lysis on the normal fibrin plates. unit of the tissue activator was defined as 1 mg the standard preparation. The activities obta were plotted with the relative concentrations percentages) as abscissa in a double logarith graph either as in Fig. 7 or directly on do logarithmic paper.

The concentration of an unknown solution read on the standard curve in percentages of standard solution containing 30 units per ml. results were then converted into units per g fresh tissue in the sample. The following i example of the calculation. A solution prepare described in Section 10 from A mg (500 mg) f tissue (ovary) in a total of B (15 ml) 2-M KS gives the dilution curve II in Fig. 7. The stan curve is I. By interpolation on the standard c it is seen that the undiluted sample correspond



7. Example of the calculation of the concen-
n of the plasminogen activator in tissue
an ovary). *Abscissa:* logarithm of the con-
tion (c) in percentages of the stock solution.
ate: logarithm of the estimated activity
lard fibrin plates). Curve I: Standard pre-
on (containing 30 units per ml). Curve II:
own solution (containing 500 mg fresh tis-
15 ml). The stock solution of II has 42 per
f the activity of the standard solution. Hence
ncentration in fresh tissue is:

$$\frac{42 \cdot 15 \cdot 1000}{100 \cdot 500} \text{ units per gram} = 378 \text{ units.}$$

y to x per cent of the standard solution. The
tration in the fresh tissue is then calculated

$$\frac{30 \cdot x \cdot B \cdot 1000}{100 \cdot A}$$

per g fresh tissue.
s a disadvantage that the standard preparation
be handled with the utmost care in order to
its potency, and that it cannot directly be
luced because the activities of the pig heart
used as starting material vary from one
to another. Since the sensitivity of the fibrin
also varies, this creates a difficult problem
standard should be lost by accident. We have
o overcome this situation by investigating the
rd curves obtained throughout our investiga-
during the past 18 months. A total of 113
n curves of the standard yields an average
y of 575 at 100 per cent concentration. When
esults are arranged in groups according to
deviations from the average activity, the
in Fig. 8 is obtained. Although the deviation
in sensitivities between the different lots of fibrinogen prevents a direct comparison of individual estimations, nevertheless, a large number of determinations can give a fairly good reproducibility of the activity. A calculation of the standard deviation of an estimation performed on the fibrinogen solution as a standard substrate gives:

Mean activity calculated $= 575$; $n = 113$; $\Sigma d^2 = 860580$; $\sigma = 87.6 \simeq 15$ per cent of the mean activity.

An estimation of the activity on a large number of the fibrinogen preparations enables us therefore to establish a new standard with very near the same activity as that used now if this should be necessary. The large individual variations in acti-

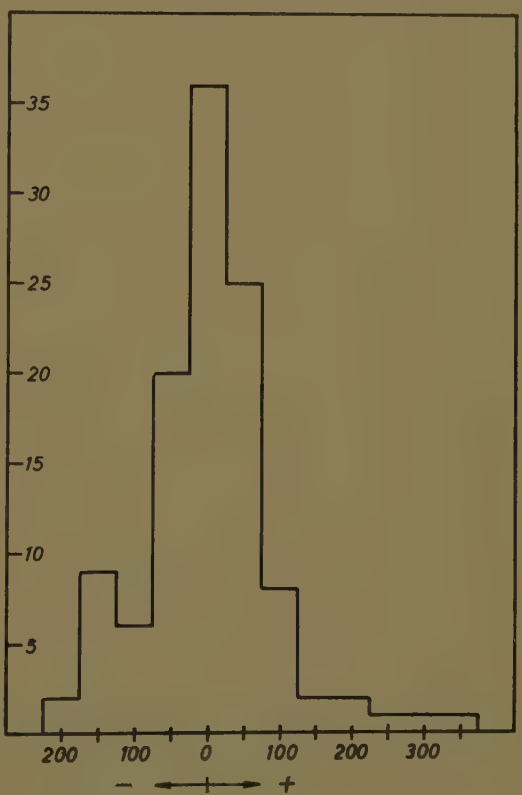


Fig. 8. The deviation of 113 standard curves expressed as the deviations of the undiluted solutions from the calculated average activity. The columns represents number of curves in groups deviating 50 in activity from each other.

vity in the tissues mean that the error in establishing a new standard preparation in this manner will be insignificant in comparison with the biological variability.

DISCUSSION

Since the discovery of the plasminogen activator in animal tissues by Astrup & Permin (1947) several attempts have been made to obtain a quantitative estimate of its concentrations in different organs. Permin (1947, 1950) made a preliminary investigation by placing small pieces of tissues direcly on the fibrin substrate in the Petri dish and measuring the lyzed zone after 20—24 hours of digestion. Powders were prepared by treating the ground tissue with acetone and these were also placed directly on the fibrin plates. Macfarlane & Biggs (1948) studied the fibrinolytic activities of saline extracts of tissue. However, the tissue activator cannot be extracted quantitatively with saline. Tagnon & Petermann (1949) and Tagnon & Palade (1950) suspended the tissue in 30 per cent sucrose and isolated an active sediment by differential centrifugation. This procedure yielded only qualitative information. Lewis & Ferguson (1950) used differential centrifugation of saline tissue suspensions. Saline extracts were also used by Fantl & Fitzpatrick (1950, human brain) and Astrup & Sterndorff (1952, pig brain and human lung). Astrup (1951 a, b) treated solutions of bovine plasminogen with dehydrated tissue powders and estimated the fibrinolytic activity formed. This was found to yield information about the activities of the tissue preparations used but a quantitative estimation was not attempted. Bierstedt (1955) extracted human tissues with a solution containing $MgSO_4$. The observations, Astrup & Stage (1952), that concentra solutions of KSCN bring the tissue activa into a solution from which it can be prec tated at acid reaction, Astrup & Sterndo (1956) made possible the development the method described here for the qua tative estimation of the tissue activator. method is selective for the tissue activ and incorporates a simultaneous estimat of certain trypsin inhibitors.

SUMMARY

1. A method for the quantitative esti tion of the plasminogen activator in tissu described.

2. The tissue activator is extracted potassium thiocyanate, precipitated at reaction, redissolved and estimated on vine fibrin (containing plasminogen).

3. Protase inhibitors, such as the ox inhibitor and the urinary inhibitor, do interfere with the estimation of the ti activator and can be estimated separatel the acid supernatant. The soy bean try inhibitor interferes with the estimations

4. Fibrinolytic components in blood labile activator and the labile inhibitor) not interfere with the estimation.

ACKNOWLEDGMENTS

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THE VARIATIONS IN SERUM URIC ACID DURING 24 HOURS AND FROM DAY TO DAY*

By B. ZACHAU-CHRISTIANSEN

From the Department of Medicine, Sundby Hospital, Copenhagen, Denmark

(Received for publication March 13, 1957)

The variation in the uric acid level in the blood or in the serum throughout a 24 hour period was followed by Lucke (1927), Joffe (1935) and Galinowski (1936, a) employing colorimetric methods and by Brøchner-Mortensen (1937) employing his own titration method. The groups of experimental subjects were small and the results lacked conformity but the highest values appeared to have a tendency to occur in the morning, falling in the course of the day and evening hours and rising at night corresponding to the rise in blood uric acid levels found by Lennox (1925) and Galinowski (1936 b) in fasting subjects. The variations comprized 1—2 mg per cent.

On account of the lack of conformity of the results, the author considered it significant to investigate whether, by employing a specific method of analysis for uric acid, a definite pattern for the variation mentioned could be discerned. This is of practical significance, *e. g.* in out-patient practice when a fixed time for the withdrawal of the blood samples cannot be arranged.

As Lassen (1954) found that the values of uric acid in the serum within a period of 2½ weeks did not vary by more than a ma mum of 1 mg per cent corresponding to variation mentioned above the experimer subject was kept on a purine-free diet, author of the present paper considered desirable, in addition, to follow this va tion from day to day when the experimer subjects received a more unrestricted diet

METHOD

As in a previous work from this hospital (L & Sylvest, 1954), the serum uric acid was de mined by the enzymatic spectro-photometric met as described by Prætorius (1949) and Prætoriu Poulsen (1953).

Double determinations were undertaken on e sample of serum and the results are given to decimal places, the second decimal being corre to 0 or 5. In the present paper, the mean of double determinations is employed. Based on double determinations, the error of the metho determined by calculation of the standard devia of the individual determinations as 0.07 mg per (see Hald, 1952). All the samples were investig by one and the same individual.

The experimental subjects received ordi hospital diet with the exclusion, however, of ticularly purine-rich foods. Ordinary meat and dishes were served and the purine content of v tables was ignored. None of the experimental jects received alcoholic beverages during the e riment. The blood samples were, in all cases, w drawn prior to the corresponding meal. Non the experimental subjects had symptoms of go

* This research was carried out with the support of a grant from The Danish National General Scientific Fund.

RESULTS

A. Serum uric acid values were determined at approximately 12 noon, 5 p.m. and 10 p.m. and at approximately 7 a.m. and noon the following day in 11 males between the ages of 16 and 47 years (average 28 years) with healthy kidneys. These patients were confined to bed with simple fractures of the extremities (9) or lesions of the menisci (2). The fact that the patients were confined to bed excludes the possible influence of muscular exertion on the concentration of uric acid in the serum. The literature on this subject has been reviewed by Poulsen (1956). Similarly, the serum uric acid was determined at approximately 12 noon, 5 p.m., 9.30 p.m. and 7 a.m. the following day in 6 male patients with healthy kidneys suffering from peptic ulcers; 3 of these patients received meatless diets while the remainder received purée diets. The ages varied between 16 and 57 years (average 39 years). The results are given in Table I and for the first and more homogeneous group also in Fig. 1. This figure shows that no immediate uniformity exists in the variation in the various time intervals between the two noon values in the 11 patients with fractures. Of these, 7 showed a maximum value at noon, 4 on the first day of the experiment and 3 on the second. It will, however, be observed that if the one noon value is a maximal value, the corresponding other noon value is approximately always very near the minimal value which reduces the variation value of the observation considerably. Two of the patients showed maximal values in the mornings.

In the second group of patients, 4 showed maximum values at noon and 2 in the evening.

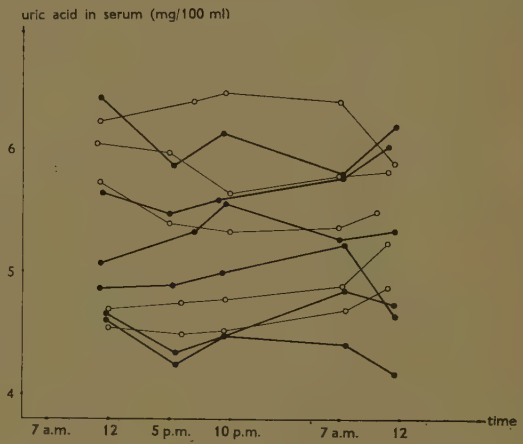

Fig. 1. The 24-hour variations of serum uric acid in 11 men with healthy kidneys.
(The corresponding values for the times and serum uric acid concentrations are indicated. The points for each individual are connected with straight lines. The differences in the marking of the points and the thickness of the lines were made solely to facilitate orientation).

In both groups, the average values at the various times are given in the table. The figures deviate only slighlty from one another and scarcely permit any generalized conclusions.

B. Subsequently, the serum uric acid concentration at about 8 a. m. on 6 consecutive days was determined in 5 male fracture patients between the ages of 17 and 54 years (average age 42 years) and in 4 peptic ulcer patients, 2 of whom received a meatless diet, between the ages of 17 and 65 years (average age 41 years). The results obtained are given in Table II. The variations from day to day in the two groups constitute, on an average, 0.45 mg per cent (0.07—0.78 mg per cent) and 0.54 mg per

Table I. *Variation of uric acid in serum during 24 hours.*

Patient	Group	Uric acid in serum (mg per cent) 12 a.m.	5 p.m.	91/2 p.m.	7 a.m.	12 a.m. following day
PA	fracture	5.08	5.33	5.58	5.28	5.33
RHC	—	5.65	5.48	5.60	5.78	6.03
FPH	—	4.65	4.35	4.48	4.40	4.18
HL	—	6.05	5.98	5.63	5.80	5.85
LLL	—	4.70	4.75	4.78	4.88	5.25
JM	—	6.43	5.88	6.15	5.80	5.85
JMC	—	5.73	5.40	5.35	5.38	5.50
VM	—	4.55	4.50	4.53	4.70	4.88
CP	—	4.88	4.90	5.00	5.25	4.45
OVP	—	4.63	4.23	4.48	4.85	4.55
ERo	—	6.23	6.40	6.48	6.40	5.90
Average		5.33	5.20	5.28	5.32	5.28
PG	ulcer-diet without meat	5.48	5.28	5.15	5.08	
SÅJ	ulcer-diet	4.35	4.60	4.45	4.45	
MK	—	4.38	4.25	4.38	4.35	4.18
OPL	ulcer-diet without meat	5.75	5.58	5.05	5.40	
KHO	—	4.78	5.18	5.30	4.95	
RP	ulcer-diet	4.05	3.93	3.93	3.93	4.08
Average ulcerpatients		4.80	4.80	4.71	4.69	

Table II. *Variation of uric acid in serum from day to day.*

Patient	Group	Uric acid in serum (mg per cent) 1. day	2. day	3. day	4. day	5. day	6. day
SSH	fracture	3.53	3.05	2.88	2.75	3.03	2.85
PVJ	—	5.78	5.28	5.50	5.60	5.38	5.43
NBK	—	4.95	5.00	4.98	4.93	4.93	4.93
VM	—	4.23	4.40	4.13	4.28	4.48	4.15
JPO	—	4.25	4.18	4.20	3.83	4.40	4.40
KPG	ulcer-diet without meat	5.38	5.30	5.70	5.30	5.03	4.90
NCJ	—	4.48	4.53	4.85	4.73	4.68	4.63
ERa	ulcer-diet	4.05	3.75	3.85	3.58	3.80	3.93
LOS	—	4.10	4.25	4.48	3.95	4.45	4.33

cent (0.37—0.80 mg per cent). If the variation be expressed as a percentage of the average for the individual experimental subject, the values obtained become 11.3 per cent (1.4—25.8 per cent) and 11.3 per cent (8.0—12.4 per cent).

For comparison it may be added that t variation during the 24 hours (only one nc value, the first, being included) for t fracture group is, on an average, 0.37 per cent (0.15—0.63 mg per cent) cor sponding to 7.1 per cent (3.3—13.6

while for the 6 peptic ulcer patients
ılue found is 0.35 mg per cent (0.12—
ng per cent) and 7.1 per cent (3.0—
per cent). In the group of peptic ulcer
its, the variation in individuals on meat-
liets is not less than that in the other
its.

STATISTICAL ANALYSIS

The variations between the separate time in-
s and between the two noon values in the
re patients in Part A were investigated to see
er the variations followed any regular pattern.
he 95 per cent confidence limits for the fre-

es $\frac{\text{number of rising values}}{11}$ be determined in

eriods mentioned (the fractions are $\frac{4}{11}, \frac{9}{11}$,
$\frac{4}{11}$), it is found that 1/2 lies within these
Further, it appears that the average varia-
in the same intervals do not differ signi-
ly from 0 as regards the 95 per cent limit as
values are -1.53, $+1.43$, $+0.39$, -0.23 and
) ($f = 10$). The afternoon, evening and night
ions in the peptic ulcer patients contribute
g to the solution of the problem.
If the results in Part B are analyzed in more
it is found that the variances of the 24-hour
es in the two groups of patients may be sum-
ed as 0.0437 and 0.0322 as Bartlett's test (see
1952) gives $\chi^2 = 12.55$, *i. e.* $< \chi^2_{0.95}$ ($f = 10$)
$^2 = 10.56 < \chi^2_{0.95}$ ($f = 5$). Thus $v^2 = 1.35$
33, $f_2 = 15$), *i. e.* no significant difference.
ion $s^2 = 0.0374$ and $s = 0.19$ mg per cent.
The variations from day to day render
$14.24 < \chi^2_{0.95}$ ($f = 8$), common $s^2 = 0.0395$
$= 0.20$ mg per cent.
The common variances in Parts 2 and 3 do
eviate significantly from one another as
.06 ($f_1 = 45$, $f_2 = 51$).
Among the variations from day to day, two
ne values in the fracture group were found
ponding to the large variation in patient SSH
ie slight variation in patient NBK. The great
ion in patient SSH is due to an isolated par-
ly high value on the first day of the experi-
Review of the case-history and dietary scheme
did not show any cause for this. The patient had received roast meat on the previous day but so had the other patients in this group. But an isolated extreme value, possibly occurring because the patient did not adhere to the conditions of the experiment, cannot upset the good agreement otherwise found. In patient VM who took part in both series of experiments, there is no significant difference between the separate variances.

6. Lous and his co-workers demonstrated that the logarithms of the uric acid values for large groups of individuals are normally distributed with better approximation than the values proper (not yet published). This observation seems to be apparent from the distribution curves in the paper by Gjørup, Poulsen & Prætorius (1955) as they show positive skewness. For these reasons and as the figures recorded have shown greater deviations the greater the average value for the individual experimental subject, the author of the present paper has, in addition, carried out a statistical analysis with the logarithmic values. This merely confirms the statements in Part 1 and 2 but the deviating variations in patients SSH and NBK become accentuated as a great variation with a particularly low level and a particularly slight variation with a medium average value are concerned. The conclusion in Part 3 is therefore not permissible but the other 7 variations are still not significantly different from the 24 hour variation and those in patient NBK are significantly less.

7. The average values for the serum uric acid $\pm$ the standard error for the 15 fracture patients and the 10 peptic ulcer patients recorded here are, expressed in mg per cent, 5.05 ± 0.21 and 4.65 ± 0.17, respectively. These average values are not significantly different as $t = 1.48$ ($f = 23$).

CONCLUSION

The 24-hour variation of serum uric acid corresponds to that which can be found from day to day; it amounts to approximately 0.8 mg per cent as a maximum and is, on an average, 0.4 and 0.5 mg per cent. No regular pattern can be discerned for the variations in the various time intervals of the 24 hours.

DISCUSSION

By means of a specific method of analysis for uric acid the statements recorded previously that the uric acid variations in different individuals show individual variations has thus been confirmed. This investigation revealed a greater tendency to maximal values at noon than in the morning in contrast to the high morning values previously postulated.

On account of these conditions and the slight quantitative variation it does not appear necessary to adhere to a definite time for the withdrawal of blood samples. Blood should, however, not be withdrawn in the period immediately after a meal. The quantitative alteration of the serum uric acid concentration caused by the various types of meals and muscular exertion will be dealt with later.

SUMMARY

Employing Prætorius' enzymatic spectrophotometric method, the variations in the serum uric acid concentration were determined during 24 hours and from day to day in males with healthy kidneys.

A tendency to the highest concentration in the forenoon was found but, in accordance with previous authors, no regular pattern for the 24-hour variations could be found. These variations are of the same order as the variations observed from day to day for 6 days and quantitatively they constitute, approximately, 0.5 mg per cent when the patients receive ordinary diet, not rich in purines.

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INFLUENCE OF PRESSURE LOSS OF VELOCITY ON PULMONARY ARTERIAL PRESSURE RECORDED THROUGH A HEART CATHETER

By B. J. JONSSON

From the Department of Clinical Physiology, Karolinska sjukhuset, Stockholm, Sweden

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right heart catheterization the pressure
ne pulmonary artery is recorded through
catheter usually with the opening lying
ne direction of the blood flow. Theoretic-
pressure thus recorded should be lower
the pressure recorded through a catheter
the opening in the opposite direction,
against the blood flow. The magnitude
ne pressure loss of velocity is in direct
ortion to the density of the blood and
quare of the velocity (v). For water the
of velocity is $\frac{v^2}{2g}$.

he significance of the velocity of flow for
sure recording in the cardiovascular
em has been discussed in the literature.
n, Jørstad & Retterstøl (1955) could
find any significant difference between
sures recorded against the blood flow and
ne blood flow direction in the femoral,
hial and radial arteries. On the other
the effect of the velocity can be very
ed in pathological conditions, as in the
c root in aortic stenosis (Castenfors,
é & Rudewald, 1954) and in the pul-
ry artery main stem in pulmonary steno-
vithout right to left shunt (Kjellberg,
nheimer, Rudhe & Jonsson, 1955;
r, Loogen & Wolter, 1954).
the pulmonary flow is much increased, as in atrial septal defect, a small pressure gradient is usually found between the right ventricle and the pulmonary artery and it might be explained by the high velocity of flow (Kjellberg *et al.*, 1955).

This paper deals with the significance of the pressure loss of velocity in the pulmonary artery main stem in three different groups of cases studied by means of right heart catheterization: 1) cases with increased pulmonary flow (atrial septal defect) but normal valves, 2) normal cases, 3) cases with slightly decreased flow (mitral stenosis).

MATERIAL

The cases studied were catheterized during the period 1954—1956. Included in the material are all cases (14) of atrial septal defect in which simultaneous pressure recording was made in the right ventricle and the pulmonary artery and all normal cases (8) and cases with pure mitral stenosis (10) in which such pressure recordings were made both at rest and during exercise.

METHODS

The pressure recording system and the technic used for heart catheterization has been described earlier (Jonsson, Linderholm & Pinardi, 1957). The right ventricular and pulmonary arterial pressures were simultaneously recorded through a double lumen catheter with a distance of 10 cm between the two openings. Under control of screening the tip of the catheter was placed in

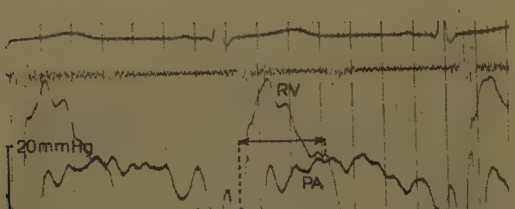

Fig. 1. Case No. 53/56. Pressure record from right atrium (RA), right ventricle (RV) and pulmonary artery main stem (PA). There is a dip in the pulmonary artery pressure during the period of maximal ejection. The arrow indicates the ejection time.

the main stem of the pulmonary artery and the proximal opening was then lying in the inflow tract of the right ventricle. During exercise the exact position of the tip was not always controlled by screening but could not be localized more than 7—8 cm distal to the ostium as the catheter w withdrawn a few cm after which a ventricu curve was obtained from the proximal openi The pressures were recorded in direct connect with the sampling of blood and respiratory air determination of the cardiac output according the Fick principle. The patients were exami in recumbent position and did not change posit during work which was performed in a ste state on a cycle ergometer. The ejection time w measured from the start of the systolic rise in pulmonary arterial pressure curve until the e of the isometric relaxation in the right ventricu pressure curve (Fig. 1).

RESULTS

In most cases with an atrial septal def a pressure gradient between the right ve tricle and the pulmonary artery was fou (Table I). The highest value was 16 mm H In spite of a very large right ventricu

Table I. *Pressure gradient between the right ventricle (RV) and the pulmonar artery (PA) at rest in 14 cases with atrial septal defect. The relative velocity of flo through the pulmonary artery can be estimated from the stroke volume and the ejection time.*

Case No.	Body surface area m²	Pulse rate beats per min.	Systol. pressure mm Hg		Pressure gradient RV-PA	Stroke volume RV ml	Ejection time RV sec/100
			RV	PA			
15/55	1.81	98	35	32	3	125	28
29/54	1.42	85	36	28	8	180	26
40/55	1.78	75	37	27	10	199	28
45/55	1.94	86	30	23	7	280	27
20/56	2.04	76	27	25	2	340	28
37/56	1.71	80	69	69	0	216	30
53/56	1.83	70	32	19	13	270	30
57/56	1.16	100	27	24	3	160	28
34/54	1.88	67	33	29	4	243	30
35/54	1.76	76	35	19	16	342	26
1/55	1.40	76	29	21	8	448	30
2/56	1.53	107	29	18	11	188	26
49/56	1.72	82	38	28	10	283	32
51/56	1.48	75	31	31	0	230	33
Mean	1.68	82	35	28	7	250	29

artery (PA) at rest and during exercise in 8 normals and 10 cases with mitral stenosis. The relative velocity of flow through the pulmonary artery can be estimated from the stroke volume and the ejection time.

			At rest					During exercise					
Case No.	Body surface area m^2	Pulse rate beats per min.	Systol. pressure mm Hg RV	PA	Pressure gradient RV-PA	Stroke volume RV ml	Ejection time RV sec/100	Pulse rate beats per min.	Systol. pressure mm Hg RV	PA	Pressure gradient RV-PA	Stroke volume RV ml	Ejection time RV sec/100
						Normals							
44/56	1.66	52	31	26	5	96	34	126	52	31	21	99	18
42/56	1.86	75	29	20	9	83	28	130	50	37	13	110	18
7/56	1.58	120	35	31	4	88	22	175	61	53	8	64	16
27/55	1.80	65	26	24	2	120	32	126	35	25	10	129	22
49/55	1.84	78	25	21	4	78	28	146	33	22	11	114	18
23/56	1.49	92	24	21	3	97	28	148	34	28	6	90	19
28/56	1.86	55	21	21	0	132	30	164	33	28	5	127	16
38/55	1.80	78	23	22	1	100	28	138	48	31	17	101	18
Mean	1.74	77	27	23	4	99	29	144	43	32	11	104	18
						Mitral stenosis							
9/55	1.78	68	60	59	1	83	30	123	125	125	0	51	22
21/55	1.68	79	85	85	0	54	25	136	135	135	0	33	20
41/55	1.62	78	21	21	0	73	29	136	38	38	0	46	18
18/56	1.86	73	23	22	1	115	30	100	47	47	0	121	26
26/56	2.03	98	61	61	0	41	22	160	98	98	0	30	18
43/56	1.78	94	78	76	2	46	22	150	106	106	0	40	16
52/56	1.93	48	45	45	3	81	28	112	100	100	0	77	28
63/56	1.74	74	35	35	0	105	32	146	63	63	0	73	22
70/56	1.74	77	28	25	3	58	28	163	54	54	0	55	16
74/56	1.65	84	38	36	2	58	26	133	56	56	0	59	20
Mean	1.78	79	48	47	1	71	27	136	82	82	0	59	21

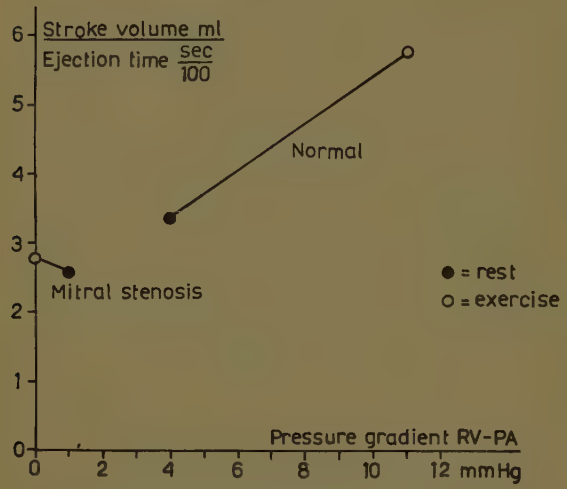

Fig. 2. The correlation between the right ventricle — pulmonary artery pressure gradient and the velocity of flow in the pulmonary artery (stroke volume/ejection time) at rest and during exercise in normal individuals and patients with mitral stenosis. The lines denote the mean values of all cases. In mitral stenosis the velocity of flow is low both at rest and during exercise and no pressure gradient was found. In the normal cases both the velocity of flow and the pressure gradient increased during exercise.

stroke volume the ejection time was as short as in the normal cases (Table II). Thus the velocity of flow must be increased in proportion to the increase in stroke volume.

Only a few of the normal cases had a significant pressure gradient at rest but during exercise it was found in all cases. The stroke volume did not change much but the ejection time was markedly shortened during exercise resulting in a higher velocity of flow.

In cases with mitral stenosis no pressure gradient was found either at rest or during exercise. The stroke volume, which was slightly smaller than normal at rest, decreased during exercise and the ejection time did not decrease to the same degree as in the normal cases. The velocity of flow was not increas during exercise. This is demonstrated in F 2, where the velocity of flow is expressed the quotient between the stroke volume a the ejection time. The body size was ab the same in the two groups.

DISCUSSION

The elasticity of the arterial wall tends transfer the intermittent output from t ventricles to a more continuous flow in t small peripheral arteries. Therefore t maximal velocity of flow is lower in t peripheral arteries than immediately dis to the semilunar ostium. At that point t velocity of flow is very high during the sh period of ejection but during the long diastole it is zero and the flow is even r versed during protodiastole. Therefore t pressure loss of velocity might influence t level of pressure recorded in the pulmona artery main stem also in normal individu although no significant effect has been fou in the peripheral systemic arteries (Cyv *et al.*, 1955).

A pressure gradient between the rig ventricle and the pulmonary artery has be found in the cases with increased pulmona flow (atrial septal defect) and during ex cise also in the normal individuals.

Can this gradient be explained by the pr sure loss of velocity when the pressure in t artery is recorded through a catheter w the opening lying in the direction of flo

It is not possible to calculate the veloc of blood flow exactly from the data availab The flow is different in the central and peripheral part of the artery and it will a vary during the phase of ejection. The of the catheter is probably most often ly in the central part of the artery but it f

e close to the wall. However, an app-
ate figure of the mean velocity during
ase of ejection can be calculated. With
umed diameter of 3 cm in the valvular
, the area will be 7.1 cm². In the cases
atrial septal defect the mean stroke
e was 250 ml and the ejection time
) sec. Thus the mean velocity of flow
e 120 cm/sec. If it were water, this
ty should imply a pressure loss of velo-

$$= \frac{120^2}{2 \times 981} \text{ cm } H_2O = 5 \text{ mm Hg.}$$

tenfors *et al.* (1954) found practically
cal pressure records in model experi-
whether water or a liquid of about the
viscosity as blood were used. The pres-
radient estimated (7 mm Hg) is there-
within the range of what was roughly
ated. These figures fit well also with
ndings of Cyvin *et al.* (1955) in model
iments. If the same calculation is made
e 35/54, in which the highest pressure
nt was found (16 mm Hg), the cor-
nding value of the pressure loss of velo-
vill be 13 mm Hg. It seems therefore
le to measure a pressure gradient of
eight in cases without a pulmonic ste-
if there is a markedly increased pulmo-
low. In such a case a different pulmo-
arterial pressure curve was obtained
the proximal opening of the catheter
sed. This opening will be directed per-
cularly to the direction of blood flow
3).

normal individuals at rest the velocity
v is usually too low to give any signi-
effect on the pressure level. The mean
ty for all cases was 48 cm/sec. giving
culated value of the pressure loss of
$y = 1$ mm Hg. The corresponding

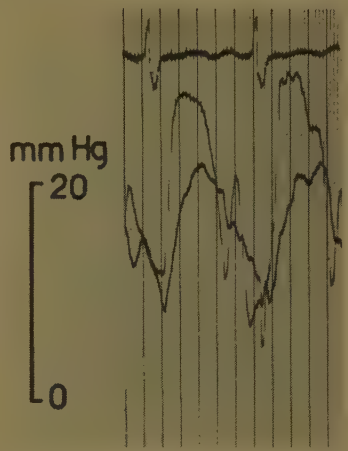

Fig. 3. Case No. 2/56. Simultaneous pressure recording with a double lumen catheter from the pulmonary artery. The curve with the lower pressure is recorded through the lumen with the opening on the tip and the curve with the highest pressure through the lumen with the opening on the shaft.

figures for the state during exercise were 82 cm/sec. and 3 mm Hg. The small pressure gradient determined during exercise in normal individuals might therefore be caused by the high velocity of flow during the very short phase of ejection. In mitral stenosis on the other hand the velocity of flow was almost as high at rest (37 cm/sec.) as during exercise (40 cm/sec.), but so low that no effect on the pressure level could be expected.

SUMMARY

The velocity of blood flow through the pulmonary artery ostium is so high in most cases with an atrial septal defect and during exercise also in normal individuals that the pressure loss of velocity should influence the pressure measurements. This seems to be the explanation of the small pressure gradient between the right ventricle and the pulmonary artery usually found in these conditions.

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ESTIMATION OF SENSITIVITY OF TUBERCLE BACILLI TO CYCLOSERINE

By A. LIND

From the Gothenburg Bacteriological Laboratory, Gothenburg, Sweden

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ycloserine (D-4-amino-3-isoxazolidone) n antibiotic effective against *Mycobac-m tuberculosis* and has been used in the c for more than two years. Cases with tant bacilli have been reported (Cum-gs, 1956; Holmdahl & Lind, 1956; er, Salomon, Reimann, Shulruff & , 1956; Moyer & Schwartz, 1956). andardized technic — as far as possible or the estimation of the sensitivity of rcle bacilli to cycloserine is therefore rable. The present paper deals with odological problems for the sensitivity nations, especially with regard to the of the inoculum and the length of in-tion time before recording the results.

MATERIAL AND METHODS

e *medium* used was Löwenstein-Jensen's m.

stock solution of cycloserine (Oxamycin, k & Co.) was prepared by dissolving 0.77 g serine (calcium salt) in 50 ml of sterile distil-vater. This stock solution, containing 10,000 active substance, was sterilized by filtration gh a Seitz filter and diluted with sterile distil-vater to 1,000 and 100 γ/ml. Prior to inspis-n adequate amounts of these solutions were to the medium to contain 5—10—20—40— nl. Inspissation was carried out for 75 minutes ° C.

e *bacilli* used were the standard strain H37Rv strain (M. 6213/56) isolated from a patient not treated with cycloserine and who had acquired her tuberculous infection prior to the introduction of cycloserine in Sweden.

Three different *sizes of inocula* were tested: 0.0001 mg, 0.01 mg and 1 mg "wet weight" in 0.1 ml suspension. The bacilli were harvested from a three week old culture on Löwenstein-Jensen's medium.

The *incubation* time was from 2—6 weeks with readings every week.

The *results were recorded* as: "confluent growth", "more than 100 colonies" (not confluent), "20—100 colonies", when less than 20 colonies the actual number was counted, and "no growth".

Plan of experiments. With each strain four parallel experiments were conducted, each experiment comprising three series, one for each inoculum and with four controls for each series, distributed in between the different concentrations of cycloserine (5—20 γ/ml).

RESULTS AND DISCUSSION

As the results in the four experiments with each of the two strains tested were mainly the same, only one experiment with H37Rv is reported here. (Fig.).

It is evident from the figure that the growth of the bacilli is to a large extent dependent on the size of the inoculum as well as on the period of incubation. With 0.0001 mg bacilli no confluent growth is seen in the controls even after 6 weeks incubation, with 0.01 mg as well as with 1 mg bacilli confluent growth is visible already after 2

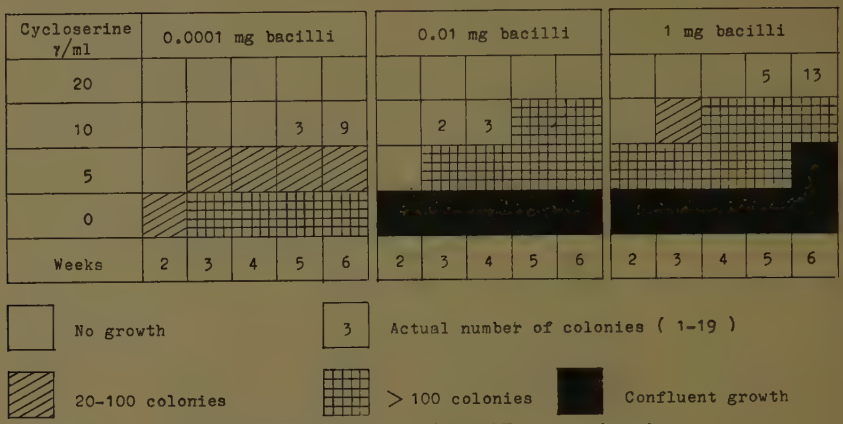

Fig. Sensitivity of the strain H37Rv to cycloserine at varying concentrations of cycloserine, inocula and times for reading.

weeks. The lowest concentration of cycloserine giving »evident inhibition« (maximal 19 colonies) was four times higher after 6 weeks incubation using an inoculum of 1 mg bacilli as compared with 0.0001 mg with an incubation period of two weeks, and twice as high when compared with the 6 weeks growth. If the result of a sensitivity estimation of a strain is expressed as "resistance ratio" (British Medical Research Council Isoniazid Trial, 1953). — compared with the H37Rv strain — the ratio is not changed more than one step for a change in the inoculum of 10,000 (within the range 1—0.0001 mg) when the growth is recorded at equal periods of incubation. Possibly the ratio can differ with more than one step if still lower inocula are compared with 1 mg bacilli.

Thus, unlike isoniazid (Middlebrook, 1956) the antibacterial effect of cycloserine is affected by the size of the inoculum. In this respect cycloserine seems to be comparable to PAS (Lehmann, 1951) and streptomycin (Middlebrook, 1954).

However, in estimations of sensitivity a great number of strains it is very la rious to standardize the inoculum by wei ing. Therefore, in routine work, the inocul has been prepared in the following way:

A representative loopful obtained from parts of a culture on Löwenstein-Jens medium was ground with a platinum l against the inside of a small (bijou) scr capped bottle containing 0.5 ml sterile di led water and a few glass beads (Bri Medical Research Council Isoniazid T 1953). The bottle was then shaken in a la ratory shaker for 30 minutes. The turbi was controlled to be approximately equa that of the standard strain (H37Rv) in One 4-mm loopful of the bacillary susp sion was spread over the surface of medium.

If the inoculum is prepared in this the sensitivity of tubercle bacilli to cy serine can be estimated analogous to esti tions of other antituberculous drugs. — I with this *indirect* type of sensitivity esti tion (inoculum consists of a culture)

direct estimation (inoculum consists of
hological specimen) the result should be
ared with the result obtained with a
lard strain, which shows the same
int of growth in the controls as does
ctual strain. Thus, with the indirect sen-
ty estimation the report to the clinician
een given in the following way (CS =
serine):

Example of a sensitive strain.

st concentration (γ/ml), which gives an evident
ition of growth:

	Patient strain (P)	Standard strain (S)	P/S
	20	20	1

Example of a resistant strain.

st concentration (γ/ml), which gives an evident
ition of growth:

	Patient strain (P)	Standard strain (S)	P/S
	80	20	4

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PORTAL VENOUS PRESSURE IN CHILDREN

By Å. GYLLENSWÄRD and M. MICHAËLSSON

From the Pediatric Department, University University Hospital, Uppsala, Sweden

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Interest in portal venous surgery has increased rapidly in recent years, especially in connection with hypertension in the portal system. A number of surveys have been published regarding portal venous pressure in normal and pathological cases. Several methods have been tried. Before 1949, the portal venous pressure was measured during laparotomy by direct puncture of the portal vein or one of its tributaries. The pressure values obtained show considerable variation and the highest value accepted in normal adults varied between 7 and 22 mm Hg in different papers (Blakemore & Lord, 1945; Taylor & Egbert, 1951, and others). These great differences may be explained by the changes that occur in the intra-abdominal pressure during anesthesia and when opening the abdomen. It is of importance that various pressure recording techniques were used. It is also worth mentioning that only a few of the authors have given reference to a baseline.

Other methods have been tried making use of the experience gained in pressure recordings in clinical cardiology. Attempts to use collaterals of the portal venous system to gauge the portal pressure have not led to routinely useful methods. Nor is it possible by this technique to diagnose cases of suspected portal hypertension, where no col terals are developed.

Myers & Taylor (1951) found that it possible to measure the portal pressure way of a catheter passed into and occludi a hepatic vein. The principle is the same that used in measuring the pressure in t left auricle through a pulmonary arte (PCV). They were able to show the practi usefulness of the method in cats. The av age difference between the simultaneou measured pressure in the portal vein and the occluded or wedged hepatic vein (OHV or WHVP) was 0.6 mm Hg. Friedman Weiner (1951) obtained similar results dogs. The first series in man seemed to ver the good correlation (Paton, Reynolds Sherlock, 1953). Since then some pap have been published where a very good agr ment has been found between the simultan ously measured pressure in the portal v and the occluded hepatic vein in patie during operation (Atkinson & Sherlo 1954).

In 1954 Atkinson & Sherlock presen a new method, where the pressure is me ured by direct puncture of the spleen. A sig ficant and linear correlation in 24 adults v found at operation between the splenic pr sure, the OHVP and the portal venous pr

(PVP). This technique cannot be used s the spleen is enlarged. Good co- ation from the patient is necessary and fore this method is not well fitted for ren.

ll a nother method was described by nan, Kelly, White, Coblentz & Fisher 5). They made a transhepatic percuta- s puncture of one of the greater branches e portal vein, and they report quite a ber of measurements. Unfortunately they ot describe enough details of their re- ation technique or their material to allow nparison with the results obtained with methods. The method is, however, not ble in children. An advantage of the two nentioned methods is that the pressure easily be recorded repeatedly in a pa-

fairly comprehensive review of the ear- terature can be found in Krook's (1956)

me years ago we saw a child with he- cirrhosis and discussed the indication aking a portal-caval shunt. The OHVP 12 mm Hg. The patient had a history o bleeding periods and varices in the agus were visible on X-ray examin- We had expected a somewhat higher ure and the surgeons consulted were not sure that a portal hypertension was really present. We could find no investigation of the portal pressure in normal children and from the available literature on adults, a pressure of 12 mm Hg could not be considered as a pathological value. Therefore, a study of the portal pressure in children without liver disease seeemed necessary in order to determine the normal range.

METHODS

A catheter was inserted through a vein in the left fossa cubiti. The pressure was measured with a strain gauge manometer in combination with a carrier-frequency amplifier (manufactured by the Elema Co., Stockholm) and the curves were registered on a direct-writing electrocardiograph with jet recorders (Mingograf, Elema Co.). The anterior axillary line with the patient in supine position was chosen as a baseline. To get comparable recordings from OHVP and free hepatic venous pressure (FHVP) the catheter was withdrawn from OHVP to FHVP during continuous pressure tracing (Fig.1). In this way the difference between these two pressures can be estimated with sufficient accuracy. When the tip of the catheter occludes the hepatic vein it is as a rule not possible to aspirate blood and this has served as one sign of the location of the catheter.

It is important that the patient is relaxed and calm; otherwise the pressure recorded may be elevated (Fig. 2). The smaler the child, the more the respiration affects the portal venous pressure,

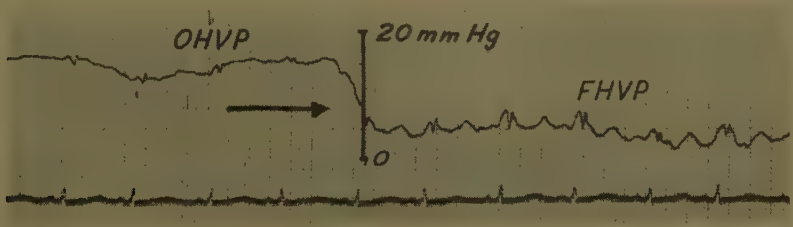

Fig. 1. Continuous pressure tracing from the hepatic vein during withdrawal of the catheter from occlusive to free position in a case of portal hypertension.

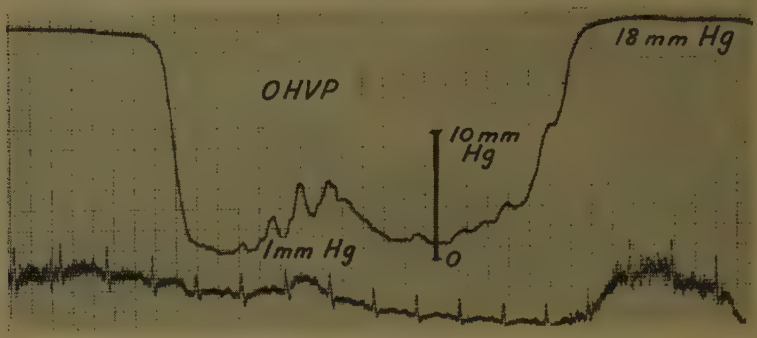

Fig. 2. Variations of the OHVP in a normal case when anxious.

and the pressure recordings must be almost simultaneous in OHVP and FHVP.

As a rule the patients have received a small dose of barbiturate before the investigation. Patients under five years of age and less got a rectal basal anesthesia with barbiturate (narkotal, Astra).

The pulse amplitude in OHVP is small and presents no problem in the pressure determination from the curve. In FHVP the amplitude is one to three mm Hg and the mean pressure was obtained by damping of the circuit.

MATERIAL

The material consists of 32 children between 3 and 14 years, who have all been catheterized on account of congenital heart disease or suspected heart disease. We have only used cases without any signs of liver disease and with normal venous and right atrial pressures. We have also had the opportunity to record the OHVP in six cases of liver cirrhosis. In three of them, the OHVP was determined twice with an interval of a half to one and a half years. All six cases were in a bad state with respect to their liver function and all of them had collateral veins and bleedings as clinical signs of portal hypertension. In two of them the diagnosis has been verified at autopsy and in the rest by liver biopsy. Two cases of extrahepatic occlusion of the portal vein have also been investigated.

RESULTS AND DISCUSSION

Some papers have been published concerning the occluded hepatic venous pressure in normal adults and in those with por hypertension (Paton, Reynolds & Sherlo 1953; Balfour, Reynolds, Mikkelsen, Pattis & Hales, 1954; Taylor & Myers, 1956). 1956, Krook published a series of 35 norm cases, where he found OHVP 5.5 mm H and FHVP 3.5 mm Hg. In 73 patients wi liver cirrhosis the pressures were 19.8 a 6.7 mm Hg respectively. These results a in good agreement with those of the abo mentioned investigations. He has used esse tially the same methods as we. Thus, material is well fitted for a comparison wi ours to determine whether there are any r differences between children and adults. preliminary report of our studies was giv in 1956 (Gyllenswärd & Michaëlsson, 195

As is seen in Table I, both OHVP a FHVP are somewhat lower in the childr and the range is larger in the adults. The d ference is statistically significant on the 1 p cent level. We should be allowed to consi our cases as normally distributed and t calculate the standard deviation. The up normal limit ($+2 \times SD$) for OHVP 6.5 mm Hg in children against 9.6 mm in Krook's series.

Table II shows that also in the cirrh

Table I. *Pressure recordings (mm Hg) in 32 children without symptoms or signs of liver disease. Krook's recordings from 35 adult controls in parenthesis.*

	Mean±2 SD		Range	
Occluded hepatic venous pressure (OHVP)	4.3±2.2	(5.5±4.1)	3—6	(2—11)
Free hepatic venous pressure (FHVP)	2.3±2.8	(3.5±2.7)	0—5	(2— 7)
Difference between OHVP and FHVP	1.9±1.5	(2.1±3.6)	0—3	(—1— 7)
Inferior vena cava pressure	2.3±3.2		0—6	
Right auricular pressure	1.5±2.8	(1.7±3.2)	0—4	(—2— 5)

Table II. *Pressure recordings (mm Hg) on 9 occasions in 6 cases of liver cirrhosis. Krook's results from 73 cases in parenthesis.*

	Mean±2 SD		Range	
Occluded hepatic venous pressure (OHVP)	11.6±5.7	(19.8±11.2)	7—15	(6—34)
Free hepatic venous pressure (FHVP)	2.7±2.7	(6.5± 6.8)	0— 4	(1—15)
Difference between OHVP and FHVP	9.3±4.3	(13.3±10.4)	7—12	(2—24)
Inferior vena cava pressure	4.1±4.8	(5.9± 6.4)	1— 9	(—2—13)
Right auricular pressure	2.7±5.5	(2.6± 5.6)	0— 6	(—3—13)

here are lower pressures in the child-
owever, our material is here too small
mit any definite conclusions although
dency is clear.

In the interpretation of the possible causes of these differences between children and adults, the technique is a matter of special concern. As mentioned before, we have used

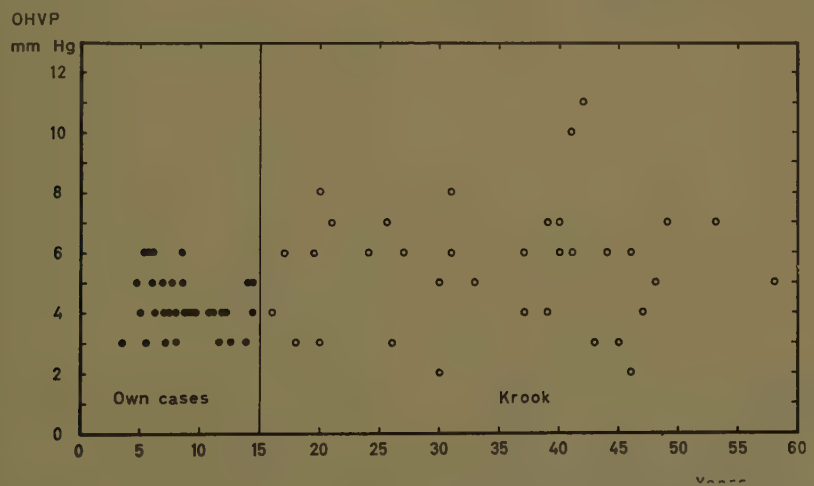

Fig. 3. The pressure in occluded hepatic vein (OHVP) in relation to age.

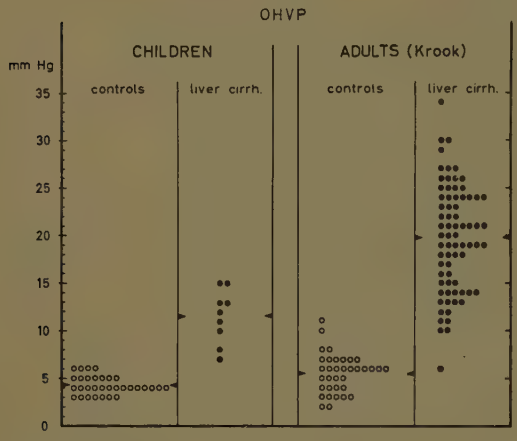

Fig. 4. Comparison between occluded hepatic venous pressure in children and adults in cases of liver cirrhosis and in control groups.

the same pressure recording equipment as Krook and we have been unable to find any systematic variation that could explain the difference. As seen in Figs. 3 and 4, the range in Krook's material is much larger than in ours, also speaking against a systematic variation in the technique. Probably there is a real difference, since Paton *et al.* (1953), Balfour (1954) and Taylor & Myers (1956) using a similar technique have also found values among adult controls which agree very well with those of Krook in occluded as well as free hepatic veins. The difference between OHVP and FHVP is the same in children and adults so it seems as though the difference may be explained by the lower venous pressure in the children. This in turn might be explained by the greater difficulty in finding really normal persons for control series among patients over 20 years.

To determine whether different locations of the catheter in the liver give different pressure recordings, we have measured OHVP at different sites in most of the pa ents but without any significant differe in pressure. One patient was tested in supi and prone position with the same OHVP both positions.

To judge the effect of the age of t patients in the OHVP, Krook's and our m terials are plotted according to age (Fig. There seems to be no correlation betwe the age and the magnitude of the OHV It seems, however, that there is a limit the age of 20. Krook has some patients und 20 years and they could just as well ha belonged to our cases, but from the age of the range is greater and the average OHV higher.

The cirrhotic patients are presented gr phically in Fig. 4. Some interesting poin might be briefly discussed. In the adu cirrhotic patients with portal hypertensi FHVP is also elevated. In the children the is no such elevation so the difference betwe OHVP and FHVP is relatively higher th in the adults. We believe that this differen is of diagnostic value in combination with t OHVP. This is especially true in cas where OHVP is at the upper normal lim Of interest is the fact that there is no ove lapping between our controls and the cas of cirrhosis. When discussing a possibl lower portal pressure in liver cirrhosis children it is worth remembering that al in other respects liver cirrhosis in childr differs from that of later life. For examp splenomegaly is more usual.

It is impossible to draw any conclusio from the 3 cases which have been catheteriz twice, since only between ½ and 1½ ye have passed between the recordings and sin they have all been splenectomized in t

ime. There are only small differences
en the two measurements. Each pres-
ecord is registered separately.
one case the OHVP was measured
two months after a portal-caval shunt
erformed and was found to have de-
d from 13 to 4 mm Hg. The FHVP
e same time had changed from 4 to
Hg.
e findings in the two cases of extra-
c occlusion of the portal vein stress the
tance of the difference in pressure be-
OHVP and FHVP rather than the
P alone. They both had an OHVP of
Hg and FHVP of 5 mm Hg, *i. e.* the
ences were within normal limits but the
dual pressures were slightly elevated.
tal hypertension of intrahepatic origin
thus be excluded. In addition it is
mentioning that the amplitude of the
re in the free hepatic vein was much
than we have seen in the other
en.

SUMMARY

naterial of 32 children, 3—14 years of
ithout any signs of liver disease and
ormal venous pressures in the inferior
ava and the right atrium, has been in-
ated with respect to the pressures in
cluded hepatic vein (OHVP), the free
c vein (FHVP) and the right atrium.
verage OHVP is 4.3 ± 2.2 mm Hg
HVP 2.3 ± 2.8 mm Hg. These values
gnificantly lower than those obtained
he same technique in adults. The dif-
e between OHVP and FHVP is
1.5 mm Hg, the same as in adults.
echnique and the results have been
discussed.

Nine recordings of these pressures in 6 cases of liver cirrhosis 8—16 years of age have also been presented. It seems as if the portal hypertension is much less extensive than in adults although the cirrhotic process is very pronounced. The difference between the OHVP and the FHVP was in all cases at least 7 mm Hg.

In two cases of extrahepatic occlusion of the portal vein the OHVP and the FHVP were slightly elevated but the difference did not exceed 3 mm Hg.

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UNEXPECTED BLOOD GROUP INCOMPATIBILITY, REVEALED BY ^{51}CR-LABELLED RED CELLS

By P. L. ADNER and S. SJÖLIN[1]

[1]the Department of Clinical Chemistry and the Department of Pediatrics, University Hospital, Uppsala, Sweden

(Received for publication April 13, 1957)

ile investigating the survival of trans- fetal red cells some interesting obser- s were made. The findings initiated a on the survival of apparently com- transfused red cells, this report giv- e first results.

METHODS

red cells were labelled with ^{51}Cr according nethod of Gray & Sterling (1950) as modi- Brante (1956). The amount of injected as 4—10 ml and the chromium content never d 1.0 μg/ml red cells. The survival curves l after autotransfusions of red cells of adults agreed very well with those published lison & Veall (1955). In our series of 12 Adner, Garby & Sjölin, 1956) the average t half life was 26 days. The activity of a taken 30 minutes after the injection was s 100 per cent.

e homotransfusions to be described here the the donors and those of the recipients were ble with respect to the ABO-group system the D (Rh$_0$) factor of the Rh system. In test the compatibility, the donor's red cells ed in saline were mixed with the recipient's

ial serological investigations including indirect test, crossmatching in albumin and also with enzyme (trypsin and papain) red cells, were made in some cases after transfusion.

th the technical assistance of S. Foconi.

Sweden this is the only type of compatibility ally performed before a transfusion.

MATERIAL AND RESULTS

Fetal red cells were obtained from the cord blood of 15 healthy, full-term newborn infants. The survival of these red cells after injection into the circulation of 15 healthy adults was studied. The recipients had never been transfused before. In 6 of these 15 experiments the survival curves were quite normal. In 4 cases the curves followed an approximate exponential course, but the life span was slightly shorter than normal (the apparent half life was 17—21 days). This finding will be further discussed in a future paper (Adner & Sjölin, 1957). In the other 5 experiments, however, the survival curves first showed a normal course, but after a time varying from 5—15 days a sudden change was evident and most of the tagged cells vanished in a short time (Fig. 1). The recipient S. F. (experiment I) has undergone four tests (Fig. 2). First he received chromium tagged fetal red cells, which showed an atypical survival curve. The survival of his own red cells tagged and reinjected was, however, normal. He also received two injections with chromium labelled red cells from an adult donor. Each time the survival curve was abnormal. The interval between the first and the second homotransfusion was 2 months and between the second and the third one, 11 months.

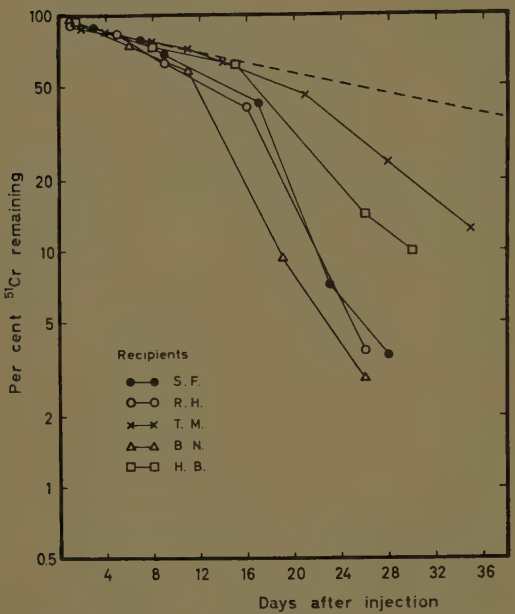


Fig. 1. Rate of disappearance of ^{51}Cr-labelled fetal red cells after injection into the circulation of 5 normal adult subjects. Dotted line: average apparent survival of autotransfused red cells of normal adults.

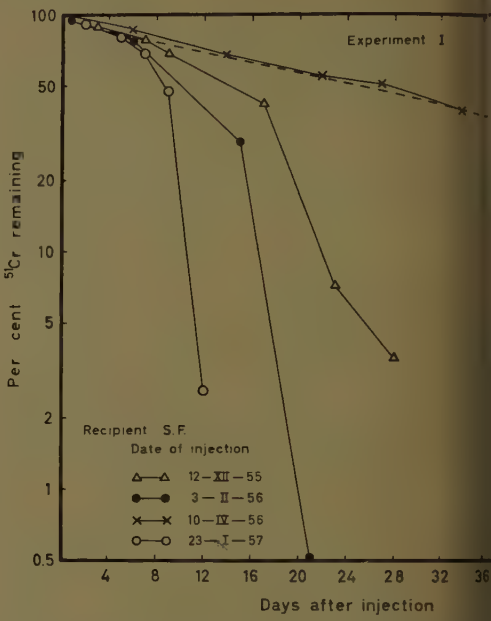
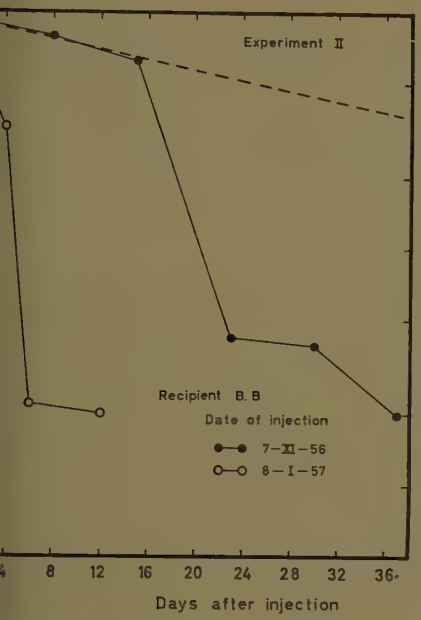


Fig. 2. Rate of disappearance of ^{51}Cr-labelled cells injected at different times into the same recipient (S.F.). Δ—Δ fetal red cells, ●—● o—o red cells from a normal adult donor, x—x the recipient's own red cells. Dotted line: average apparent survival of autotransfused red cells of normal adults.

These findings prompted us to investigate the fate of compatible red cells transfused from one healthy adult to another. Also in this series no recipient had received any blood transfusion before. In 12 of 15 such homotransfusions the survival curves were normal. In the other 3 recipients (experiments II, III, and IV), however, a sudden break in the curve was found after a time varying from 9 to 18 days. In these three cases the experiments were repeated using the same donor and the same recipient. Again abnormal survival curves were found in each case (Figs. 3, 4, and 5). In experiments II and IV, with the second transfusion following the first one after 2 and 5 months respectively, a very rapid destruction of the tagged red cells occurred. In experiment III, which 3½ months had passed between the first transfusion and the second one, the cell life span was shorter than normal, but not as short as in experiments II and IV. In Table I some pertinent serologic data are shown. In one recipient's serum (experiment II) an antibody against factor E(rh) was discovered in a sample taken immediately before the second transfusion. In another recipient's serum (experiment IV) an antibody against the Duffy system (anti Fy) was discovered 12 days after the second transfusion was given. In experiments I and III no antibodies could be detected with the serological methods used.



Rate of disappearance of ^{51}Cr-labelled red
an adult donor after injection into the cir-
of an adult recipient (B.B.) on two dif-
occasions. Dotted line: average apparent
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normal adults.

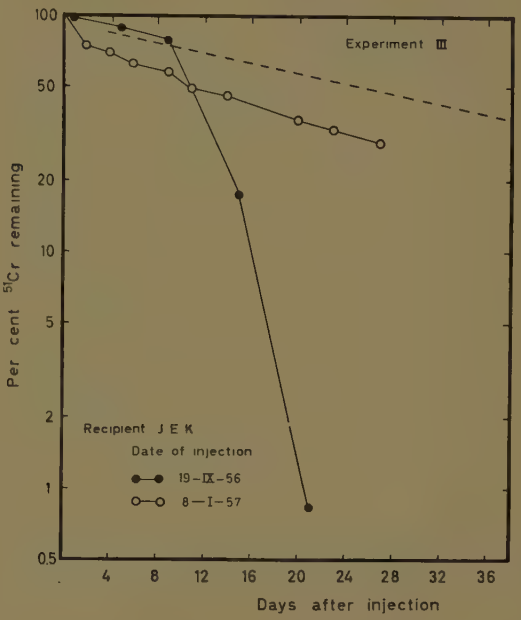

Fig. 4. Rate of disappearance of ^{51}Cr-labelled red cells of an adult donor after injection into the circulation of an adult recipient (J. E. K.) on two different occasions. Dotted line: average apparent survival of autotransfused red cells of normal adults.

DISCUSSION

ough we have not been able to detect
lies in more than two cases we are
d to interpret the course of all these abnormal survival curves as caused by immunisation. This hypothesis is further supported by the fact that the second transfusion in three cases was followed by a more

Table I

	I		II		III		IV	
	Donor	Rec. S.F.	Donor	Rec. B.B.	Donor	Rec. J.E.K.	Donor	Rec. Å.V.
	A_2	A_1	A_1	A_1	A_1	A_2	A_1	A_1
	cDE/cde Rh_2rh	CDe/cde Rh_1rh	CDe/cDE Rh_1Rh_2	C^wDe/CDe $Rh_{1w}Rh_1$	CDe/CDe Rh_1Rh_1	cDE/cde Rh_2rh	cde/cde rhrh	cde/cde rhrh
dy		none		anti-E rh"		none		anti-Fy^a

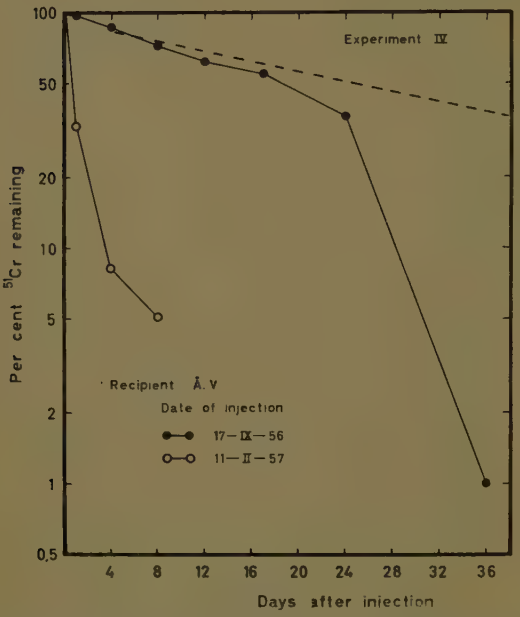

Fig. 5. Rate of disappearance of ^{51}Cr-labelled red cells of an adult donor after injection into the circulation of an adult recipient (Å.V.) on two different occasions. Dotted line: average apparent survival of autotransfused red cells of normal adults.

rapid elimination of the injected red cells than the first one. The same type of curves has been reported by Stohlmann & Schneiderman (1956) in investigations on dogs, where such curves occurred in a frequency of 8 out of 33 compatible transfusions. They did not, however, succeed in demonstrating the appearance of any antibody. Jandl & Greenberg (1957) obtained similar curves in two patients with splenomegaly. Here also the serological investigation was negative.

At present it is impossible to state exactly how often a homotransfusion induces an immunisation, but it is suggested that such a phenomenon might be more common than is usually assumed. If, in addition, the frequency of immunisation is so high as preliminary investigation indicates, most these cases obviously do not develop conspicuous clinical symptom. In any event immunisation may be the cause of the known fact that repeated transfusions recipient are often less and less effective

The radioactive chromium method seems to be a sensitive means of detecting such immunisation. With the Ashby technique a phenomenon similar to that described above seems to have been observed only (Mollison, 1951). This discrepancy perhaps be explained by the presence in immunized subjects of a relatively low body activity, which is sufficient to destroy only a few of the great number of red injected by the Ashby technique. In connection it seems necessary to mention that our knowledge of the effects of chromium on red cells is still incomplete. Besides the direct toxic effect of the metal, it must be remembered that chromium may be capable of modifying the antigenic properties of the red cells.

Finally it must be considered whether will not prove necessary to make more complete serological investigations before transfusions. It must also again be emphasized that blood transfusions should only be given in the presence of rigid indications. We hope to be able to consider further these and other important aspects of blood transfusion later communication.

SUMMARY

Thirty healthy adult subjects were given "compatible" ^{51}Cr-labelled red cells from different healthy adult and infant donors

ases abnormal red cell survival curves pecial type were found. Immunisation uspected, and in 2 out of 4 cases, all ighly investigated, a relevant antibody iscovered. The relative inefficiency of sual compatibility tests performed be- a transfusion is stressed.

ACKNOWLEDGEMENTS

the cord blood samples we are greatly in- to Prof J. Naeslund. We appreciate the co- on of Prof. B. Broman, Stockholm, who has performed the serological investigations. xpenses of this investigation were defrayed grant from the Swedish Medical Research l.

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THE EFFECT OF IODOCHLOROHYDROXYQUINOLINE, DI-IODOHYDROXYQUINOLINE AND DICHLOROHYDROXYQUINALDINE ON THE THYROID UPTAKE OF RADIO-IODINE

By H. CASTENFORS and A. M. ALLGOTH

From the 2nd Medical Department, St. Eriks sjukhus, Stockholm, Sweden

(Received for publication April 11, 1957)

Several times, while undertaking routine diagnostic procedures, we have observed that the 24-hour uptake of tracer iodine is very low in patients who had received iodine-containing intestinal antiseptics immediately before the test. As the study of this uptake often would be of value also in patients with intestinal disturbances we have carried out the present investigation in order to establish the magnitude and duration of this iodine-blocking effect. The intestinal antiseptics that we used comprised two iodinated quinoline derivatives and dichlorohydroxyquinaldine. The last-named compound was included in order to establish whether the effect is due to the quinoline molecule.

MATERIAL AND METHOD

The material consisted of 13 patients, treated in the 2nd Medical Department of St. Erik's Hospital. None of them showed any signs of hypo- or hyperthyroidism. While the study was in progress, no substances which could influence the tests were given to the patients, nor were any radiological examinations undertaken. After an initial test with the tracer, one of the intestinal antiseptics was given for 7 days in the following daily dose:

Iodochlorohydroxyquinoline	1.5 g (2 tablets t.i.d.)
Di-iodohydroxyquinoline	1.8 g (2 tablets t.i.d.)
Dichlorohydroxyquinaldine	0.6 g (2 tablets t.i.d.)

The tracer test was repeated at the end of tr ment and after further 7 days; if any effect mained after this time, the test was repeate intervals of one to two weeks for up to 3 mo

For the test, 30 μc of carrier-free I^{131} v given; correction for residual activity was alw made. The instrument used was a Geiger-Mi counter (Sensitivity: 110 counts/min/μc at a tance of 30 cm. Background 300 counts/min.) counting distance was 30 cm. The uptake was pressed in per cent of the given amount meas at the same distance in a glass beaker. The m mum deviation for repeated tests is within 20 cent of the uptake.

RESULTS

All 8 patients who had had iodochl hydroxyquinoline showed a subnormal hour uptake after 7 days' administra (Fig. 1). One or two weeks after the en treatment the uptake was higher than initial value. The results in the 3 patients had had di-iodohydroxyquinoline are sh in Fig. 2. One case behaved similarly to t in the group treated with iodochlorohydr quinoline; in the 2 reemaining patients uptake remained depressed for sev months.

Treatment with dichlorohydroxyquinal in 6 cases (Fig. 3) resulted in no effec the uptake whatever.

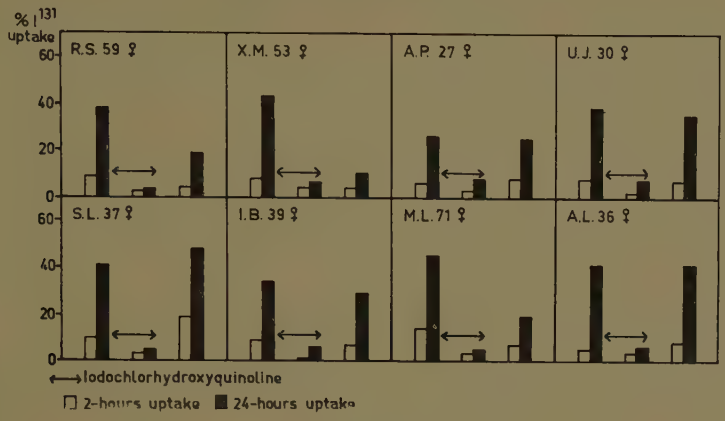

Fig. 1.

DISCUSSION

ier investigators (David, Reed & 1933; Haskins, Luttermoser & 1950; Knight & Miller, 1949, and , have found that systemic absorption ochlorohydroxyquinoline and di-iodo-yquinoline does occur, as shown by rease in blood iodine level after administration of these drugs. Iodochlorohydroxyquinoline is absorbed and excreted faster than di-iodohydroxyquinoline; and in therapeutic doses the latter gives the greater increase in blood iodine (Knight & Miller). Haskins *et al.* have shown in animal experiments that most of the absorbed iodochlorohydroxyquinoline is excreted substantially unchanged. How, then, is the effect of iodochlorohydroxyquinoline and di-iodohydroxyquinoline on the thyroid uptake of radio-iodine to be explained?

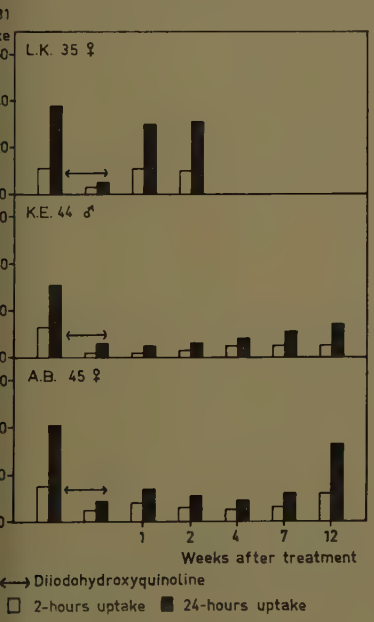

Fig. 2.

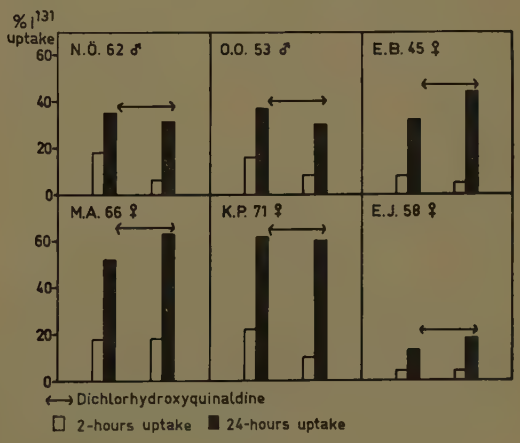

Fig. 3.

In therapeutic doses of iodochlorohydroxyquinoline and di-iodohydroxyquinoline a patient receives about 500—1000 mg of combined iodine daily. As very small amounts of iodine (5—10 mg) are known to have a blocking effect on the accumulation of I^{131}, (Stanley & Astwood, 1948) it could be suspected that even a small dissociation of the iodinated hydroxyquinoline derivatives would exert such an effect. The fact that dichlorohydroxyquinaldine does not have any effect on the thyroid uptake shows that the quinoline molecule itself has no antithyroid effect. It cannot be excluded that iodinated quinoline molecules have an antithyroid effect, but this seems unlikely. The fact that the di-iodohydroxyquinoline has had a more prolonged blocking effect than the monoiodinated substance can be explained both by its higher iodine content and by its tendency to slower excretion.

SUMMARY

A comparative study of the effect of iodochlorohydroxyquinoline, di-iodohydroxyquinoline and dichlorohydroxyquinaldine on the thyroid uptake of I^{131} was carried out. Iodochlorohydroxyquinoline and di-iodohydr quinoline in therapeutic doses were foun depress the uptake to subnormal values. blocking effect of di-iodohydroxyquino could in two cases be followed for two mo after the administration. The effect is p ably due to a slight metabolic dissocci of iodine from the organic nucleus, s dichlorohydroxyquinaldine has no eeffec the thyroid uptake.

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HE PREPARATION OF PROACCELERIN DEFICIENT (PARAHEMOPHILIA) PLASMA FOR THE ASSAY OF PROACCELERIN

By H. STORMORKEN

From the Department of Physiology, Veterinary College of Norway, and The Coagulation Laboratory, Rikshospitalet, Oslo, Norway

(Received for publication May 24, 1957)

r the assay of proaccelerin both one- and two-stage methods are used. The tage method of Ware & Seegers (1947) laborious for routine work, and prob- not more accurate than the one-stage ods.

substrate plasma in the one-stage assay an use, if available, plasma from a t with parahemophilia (Owren, 1947), ored plasmas with little or no proac- n left. Such plasmas have been made in al ways (Stefanini, 1950; Wolf, 1953; & Ware, 1953; Deutsch, 1955, and s), most methods being modifications e method of Quick & Stefanini (1948). is common experience that plasmas l according to any of these directions considerably from one bath to another. is not surprising since in none of them sufficiently controlled, and both pro- rin and fibrinogen are much influenced e hydrogen ion concentration during ge (Stormorken, 1957, a & b). Both orate more rapidly at high pH, and g fibrinogen deterioration an anti- lant is produced. For these reasons it possible to obtain a plasma by storage is exactly like the natural parahemo- plasma. On the basis of the results of ability studies, however, it was possible to select conditions where proaccelerin disappeared while fibrinogen reactivity was little affected. For this, only human plasma could be used, because the concentration and stability of proaccelerin in other species (bovine, canine and equine) were too great. Thus, too long time or a too high pH were required for the deterioration of proaccelerin, and consequently the fibrinogen denatured. However, with care, a usable preparation could be obtained from horse plasma.

Proaccelerin deficient plasma can best be made from either oxalated or EDTA plasma. Citrated plasma or plasma collected by passage through cation exchange resin (Dowex IR-120, sodium cycle) are less suitable because of the high stability of proaccelerin in these plasmas. The effect of various anticoagulants on the stability of proaccelerin is shown in Fig. 1.

METHODS

The procedure for making parahemophilia plasma artificially is as follows: Blood from a fasting donor is collected in one tenth volume of anticoagulant and centrifuged for 30 minutes at 2,500 rpm. The plasma is then dialyzed for 24 hours at room temperature in cellophane casings (Visking) against saline containing the same amount of anticoagulant as plasma, *i. e.* one sixth volume. The

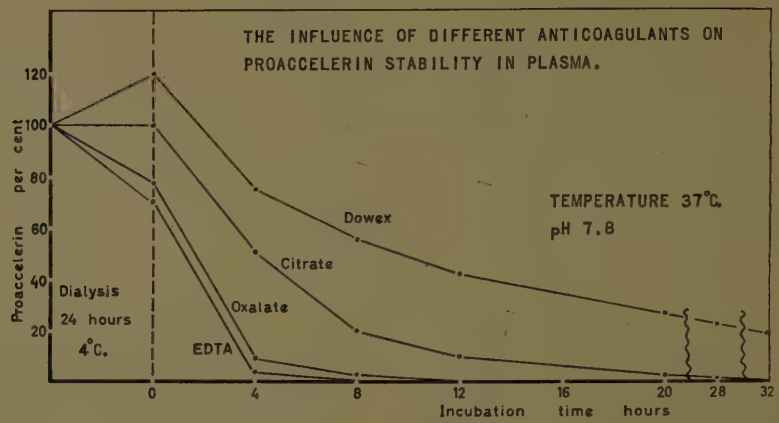

Fig. 1. The blood was drawn in one tenth volume of the following anticoagulants: Sodium citra dihydrate 3.13 per cent, potassium oxalate monohydrate 2.5 per cent, and the disodium salt EDTA (Complexion III, B. Siegfried, Switzerland), 1 per cent in physiological saline. Res treated blood was collected by passage of 50 ml through a 20 ml column of Dowex IR-120 on t sodium cycle. The specimens were treated in the same way throughout, and the incubation perform in 10 ml portions.

purpose of dialysis is to make the pH stable by removing the bicarbonate buffer system. Thereafter the pH is adjusted to 8.0 with N/2 NaOH, and the plasma placed in a water bath at 37° C. During

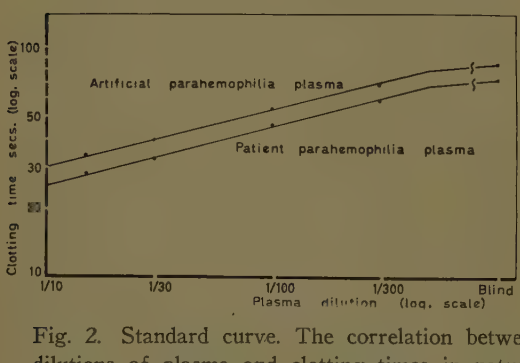

Fig. 2. Standard curve. The correlation between dilutions of plasma and clotting times in natural and artificial parahemophilia plasma. Clotting system: 0.2 ml thromboplastin + 0.2 ml parahemophilia plasma + 0.2 ml diluted normal plasma + 0.2 ml calcium chloride, optimal concentration. The dilutions were performed as described by Hjort *et al.* 1955, in which paper the compositions of the dilution fluids are also desribed.

incubation the pH should be checked once or tw and should not be allowed to drop below 7.7. plasma is left in the water bath until the throm plastin time (0.2 ml thromboplastin + 0.2 ml cubated plasma + 0.2 ml dilution fluid II (bli + 0.2 ml $CaCl_2$, optimal concentration) reaches seconds. No proccelerin can then be demonstra After adjustment of the pH to 7.4, the plasm frozen in the liquid state in small aliquots stored at —20° C. After thawing of one aliquo correlation curve is made by testing dilutions pool of normal plasmas in the above menti system. Such a correlation curve is shown in Fi where a comparison is made between natural artificial parahemophilia plasma. It is seen both correlation curves are straight lines within range 1/10 to 1/500 plasma dilution, and are al parallel. The clotting times, however, are longe the artificial system.

RESULTS

The tests of special interest in the paration of artificial parahemophilia pla are the thromboplastin time and the thr

Table I. *The changes in thrombin time and thromboplastin time of oxalated, dialyzed plasma on incubation at 37° C and pH 7.8.*

	Incubation time, hours						
	0	4	4	12	16	20	24
Thrombin time	13.4	13.7	14.0	14.5	15.1	15.6	16.7
Thromboplastin time ...	14.0	33.1	49.2	63.0	75	84	90

Thrombin time: 0.2 ml plasma + 0.2 ml veronal buffer + 0.2 ml thrombin solution. Thromboplastin time: 0.2 ml thromboplastin + 0.2 ml plasma + 0.2 ml dilution fluid II + 0.2 ml calcium chloride, optimal concentration. Clotting times in seconds.

time, which show mainly the degree of accelerin deficiency and the reactivity of fibrinogen respectively. Both prothrom- and proconvertin are sufficiently stable stand the incubation procedure, but are derately reduced. The changes in trombo- stin time and thrombin time during in- ation in an experiment with oxalated sma are shown in Table I.

The thrombin time was prolonged 3—4 onds at the time when the thromboplastin e indicated a total deficiency of pro- elerin as judged from comparison with ural parahemophilia plasma. If the pH s adjusted to 7.4 after incubation, the ombin time decreased to preincubation ue while the thromboplastin time in- ased. This finding indicates that a slug- n thrombin fibrinogen reaction is not the se of the difference in thromboplastin e between natural and artificial para- nophilia plasma. According to experi- nts by Waaler (1957) the difference is st likely caused by loss of hemophilic tors during incubation, because the nor- intrinsic clotting system affects the omboplastin time when above 25—30 seconds. Although a lowering of the pH after incubation results in longer clotting times, it is probably advantageous because of the resulting increase in stability on storage.

Further, two things deserve mentioning. 1) When EDTA is used as an anticoagulant, it is necessary to neutralize the EDTA-saline mixture before dialysis — otherwise plasma proteins are precipitated due to the low pH. 2) The time required for the disappearance of proaccelerin is to some degree dependent on the initial level of proaccelerin, on the varying anticoagulant concentration due to varying hematocrit, and on the ratio plasma volume/contact surface. With small volumes the disappearance of proaccelerin is faster, but unfortunately this is also the case with the deterioration of fibrinogen. Therefore, it is of no advantage to increase the surface with glass beads for instance. To keep the incubation time within 20—30 hours, 40—50 ml portions of oxalated plasma in tubes of the same size are suitable.

If carefully carried through, and with equally potent thromboplastin, nearly identical preparations are obtained in different experiments.

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PAPER ELECTROPHORETIC STUDY OF HUMAN FETAL SERUM PROTEINS WITH DEMONSTRATION OF A NEW PROTEIN FRACTION

By C. G. BERGSTRAND and B. CZAR

From The Pediatric Clinic at Kronprinsessan Lovisas Barnsjukhus, Stockholm, Sweden

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elatively early investigations have shown in humans as well as in some animal ies certain differences exist between fetal maternal serum proteins. The total pro- content and protein pattern are different. introduction of micro-electrophoretic hods has made possible more detailed ies of serum protein fractions even in young fetus. The results obtained have tributed to the discussion of such interest- problems as the origin of fetal serum eins and the placental passage of mater- antibodies.

arlier work in this field is reviewed by rmer (1952), Grell & Stürmer (1953) by Furuhjelm (1956). Most of the in- igations have mainly been concerned newborn infants and relatively few lies have been made on fetal sera. It is, ever, well established that the total serum ein content of the fetus is very low ing the first months and rises continually ing gestation. This is chiefly caused by an ease of albumin and γ-globulins whereas α- and β-globulins remain relatively stant during the whole period of gestation oore, Martin du Pan & Buxton, 1949; rbeck & Levens, 1950; Malmnäs, 1950; ss & Röttger, 1951; Grell & Stürmer, 3; Bang & Påby, 1955; Sternberg, Dage- nais-Perusse & Dreyfuss, 1956). At term the total serum protein value is somewhat lower than in older children and adults; the γ-globulins are found in high concentration and decrease after birth.

The purpose of the present work was originally to study in more detail the composition of the fetal serum protein by means of paper electrophoresis. This method has hitherto been little used for this purpose but is very suitable as it makes possible an analysis of the often very small quantities of serum that can be obtained from the young fetus. At an early stage of the investigation a protein component which seemed not to have been observed earlier in human fetal sera was demonstrated by the method used (Bergstrand & Czar, 1956). The later part of the work has been an attempt to obtain more detailed information of this unknown component.

MATERIAL AND METHODS

The fetuses were obtained from legal abortions carried out by caesarian section. The mothers were with few exceptions in good physical health and none of them had shown any symptoms that could possibly have had a deleterious influence on the health of the fetus. The age of the mothers ranged from 14 to 43 years.

Table I. *Error of the method determined by double estimations of 14 fetal sera chosen at random.*

Fetal serum protein fractions	Mean value per cent	Error of the method
Albumin	73.65	1.26
Substance X	9.60	0.36
α_1-globulin	3.53	0.23
α_2-globulin	3.96	0.47
β-globulin	4.48	0.28
γ-globulin	4.80	0.55

The fetuses ranged in length from 10 cm to 27 cm corresponding to a gestation period of 9 to 19 weeks.

Blood was taken from the fetus by heart puncture (right ventricle) immediately after the operation. Blood from the mothers was drawn from a cubital vein at the same time.

The blood specimens were centrifugated within a few hours. In some cases the serum was kept in the freezing compartment of a refrigator for not more than a week. Determination of total serum protein was made before freezing. The fetal sera were concentrated to about a third of the original volume by evaporation under reduced pressure.

Paper electrophoresis was carried out with the apparatus described by Dettker & Andurén (1954). The filter paper used was Whatman No. 1 and the amount of serum applied to the strip, 0.015 ml. Analyses were conducted at pH 8.6 with a diethyl barbiturate buffer of 0.1 ionic strength. The voltage was 220 v. The electrophoresis was run for 12 hours and the strips were stained with Ponceau red. The concentration of the different protein fractions was measured by spectrophotometry (Beckman, B.) after elution of each fraction with 0.01N NaOH. The total protein content of the serum was determined by the biuret method.

A total number of 39 fetuses and 33 mothers was examined. Strips with fetal and maternal sera were run simultaneously in the apparatus and the electrophoretic patterns were compared.

Sera from 11 premature babies ranging in weight from 1320 g to 2360 g were analysed as described above and compared with the fetal sera.

Double estimations were made in all cases, *i. e.* t strips were analysed and the mean value for e protein fraction was used for the diagrams. Er of the method was determined by double estim tions of 14 fetal sera chosen at random (Table

RESULTS

The amount of total protein and the re tive and absolute concentrations of the d ferent protein fractions in maternal and fe sera in relation to fetal length are given the diagrams.

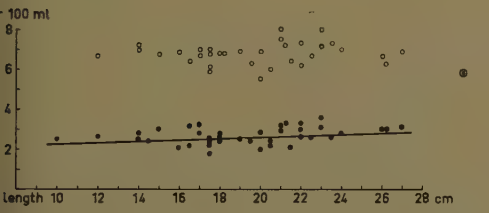
The values of the maternal sera sh rather great variations in most cases. T mean values of total protein and the d ferent fractions of maternal sera are giv in Table II. Table III shows the mean va of total protein and of the different prot fractions of fetal sera, standard error the mean, the equation of the regression l and the standard deviation from the regr sion. The significance of the slope of regression line in relation to zero line is a given in the table.

Table II. *Total protein and differ fractions in per cent of total protein and g per 100 ml of maternal serum. Num of mothers 33.*

Protein	Mean	Standar error
Total protein	6.79	0.09
Albumin per cent....	60.03	0.89
» abs.	4.07	0.07
α_1-globulin per cent..	5.61	0.28
» abs.	0.38	0.02
α_2-globulin per cent..	8.60	0.37
» abs.	0.58	0.03
β-globulin per cent..	10.48	0.40
» abs.	0.73	0.03
γ-globulin pert cent .	15.30	0.51
» abs.	1.04	0.04

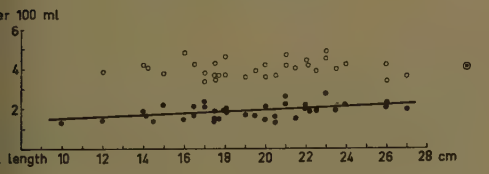
Table III. *Number of fetuses 39. x = fetal length (mean 19.3 cm). y = protein fractions in per cent of total protein and in g per 100 ml. Levels of significance: * = 1 per cent < p < 5 per cent; ** = 0.1 per cent < p < 1 per cent; *** = p < 0.1 per cent.*

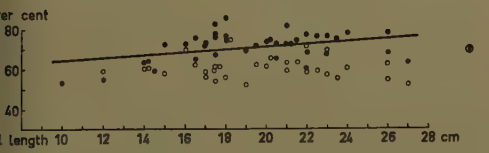
Protein	Regression line	Standard deviation from regression	Mean	Standard error	Significance
Total protein	$y = 0.035x + 2.01$	0.40	2.69	0.66	*
Albumin per cent	$y = 0.747x + 56.19$	5.83	70.58	0.93	* *
Albumin abs.	$y = 0.044x + 1.05$	0.30	1.90	0.05	* *
Substance X per cent	$y = -0.585x + 1.78$	2.13	10.50	0.34	* * *
Substance abs.	$y = -0.012x + 0.51$	0.076	0.28	0.01	* * *
α_1-glob. per cent	$y = -0.237x + 8.63$	0.83	4.07	0.13	* * *
α_1-glob. abs.	$y = -0.00093x + 0.13$	0.039	0.11	0.01	—
α_2-glob. per cent	$y = -0.045x + 5.30$	1.13	4.43	0.18	—
α_2-glob. abs.	$y = 0.00017x + 0.12$	0.040	0.12	0.01	—
β-glob. per cent	$y = -0.185x + 8.96$	1.49	5.41	0.24	* *
β-glob. abs.	$y = -0.0029x + 0.20$	0.048	0.15	0.01	—
γ-glob. per cent	$y = 0.152x + 2.06$	1.62	4.98	0.26	*
γ-glob. abs.	$y = 0.0063x + 0.02$	0.053	0.14	0.01	* *



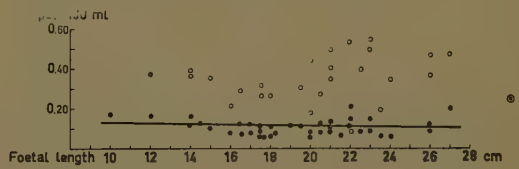
. 1. Total protein in g per 100 ml. Maternal serum: o Fetal serum: ⊙ Mean value of 11 premature babies: ⊙

The first diagram (Fig. 1) shows total protein of maternal and fetal serum. There is, as seen from the diagram, in the fetus a tendency towards increasing serum protein with increasing length and the slope of the regression line is almost significant.





. 2. Albumin in g per 100 ml serum and in er cent of total protein. Symbols as in Fig. 1.



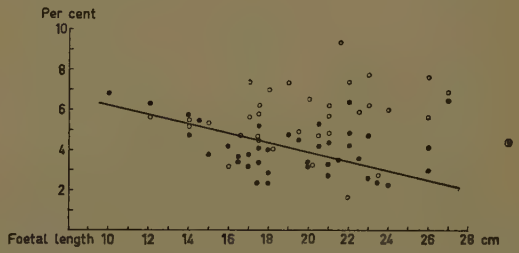


Fig. 3. α_1-globulin in g per 100 ml serum and in per cent of total protein. Symbols as in Fig. 1.

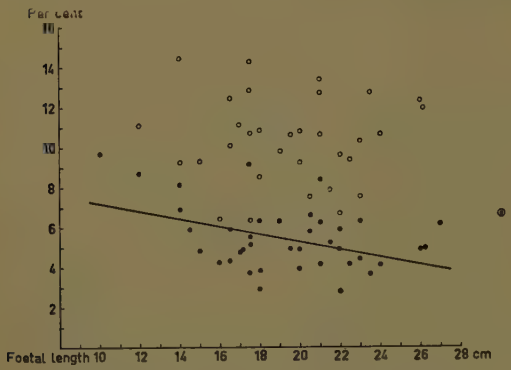


Fig. 4. β-globulin in per cent of total protein. Symbols as in Fig. 1.

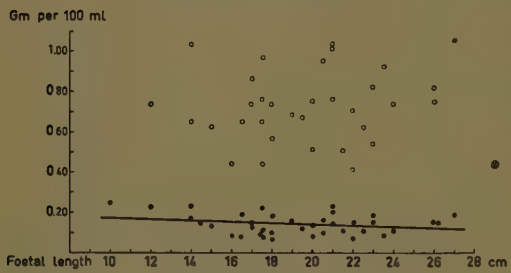
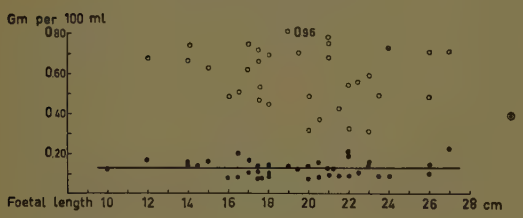


Fig. 5. β-globulin in g per 100 ml serum. Symbols as in Fig. 1.



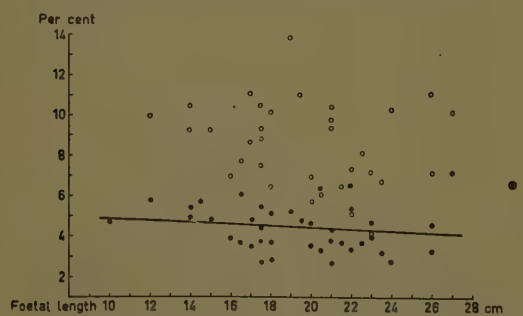


Fig. 6. α_2-globulin in g per 100 ml serum and in per cent of total protein. Symbols as in Fig. 1.

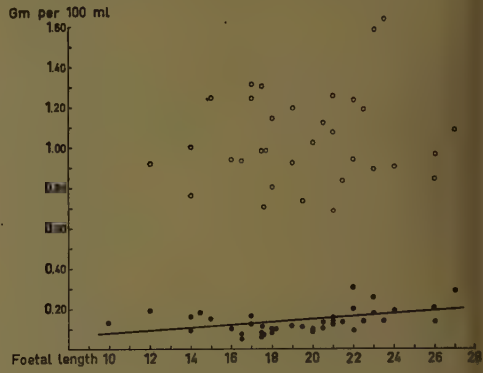


Fig. 7. γ-globulin in g per 100 ml serum. Symbols as in Fig. 1.

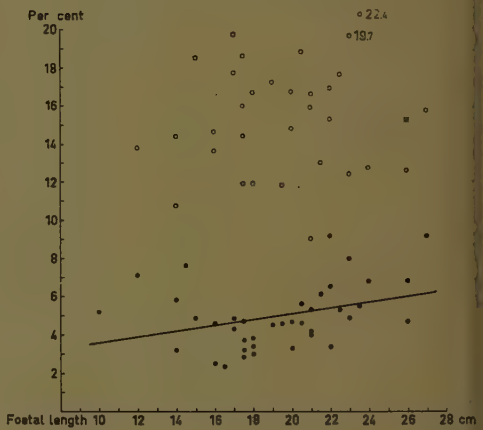
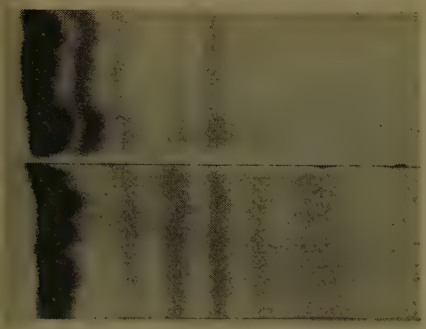


Fig. 8. γ-globulin in per cent of total protein. Symbols as in Fig. 1.

Table IV. *Relationship between mater serum protein fractions expressed in per c of total protein and corresponding fetal fr tions. r = correlation coefficient. Levels significance as in Table III.*

Protein fraction	r	Significan
Albumin	0.40	*
α_1-globulin	0.21	—
α_2-globulin	0.44	*
β-globulin	—0.95	* * *
γ-globulin	0.22	—



9. Top: Fetal serum. Bottom: Maternal serum. Fetal length 14 cm.

During the observed period of gestation l albumin increases both relatively and olutely (Fig. 2). The percentage of fetal globulin shows a marked decrease (Fig. and the same holds true for the fetal β bulin (Fig. 4). A change in the absolute ounts of these fractions is, however, not monstrable (Figs. 3 and 5). Fetal α_2-glo- in does not seem to change during the erved period (Fig. 6).

The absolute amount of fetal γ-globulin reases (Fig. 7) and the relative amount his fraction shows an almost significant rease (Fig. 8).

A relationship between maternal and fetal tein fractions is difficult to predict from diagrams. The maternal serum protein tions expressed as a percentage of the l protein were related to the correspond- fetal fractions and the correlation coef- ents were calculated. The coefficients and r significance are given in Table IV. re is, as seen from the table, a possible relation in the case of albumin and α_2- bulin. A definite inverse correlation exists ween maternal and fetal β-globulin.

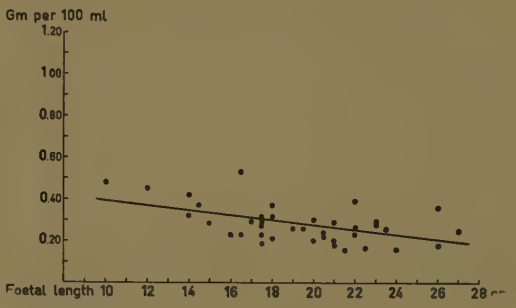


Fig. 10. Substance X in g per 100 ml fetal serum.

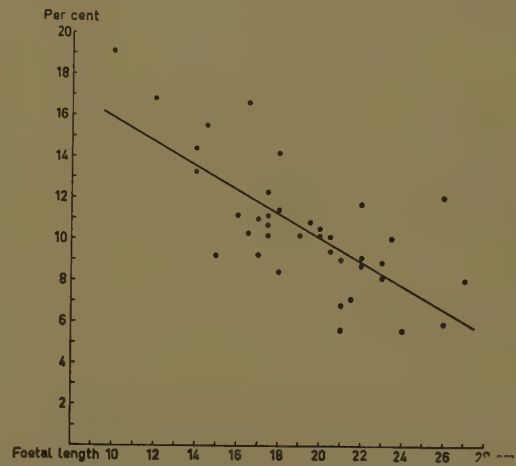


Fig. 11. Substance X in per cent of total fetal serum protein.

In all the fetal sera, as mentioned above, a protein fraction is found which is not observed in maternal serum or in serum from premature babies. It is clearly seen in the paper strip as a separate band between the albumin and the α_1-globulin and has in the present paper been called "substance x" as its true nature is not clear (Fig. 9). The concentration of this component is considerable, amounting to about 10 per cent of the total serum protein. The highest relative and absolute values are found in the youngest fetuses and a significant decrease takes place during

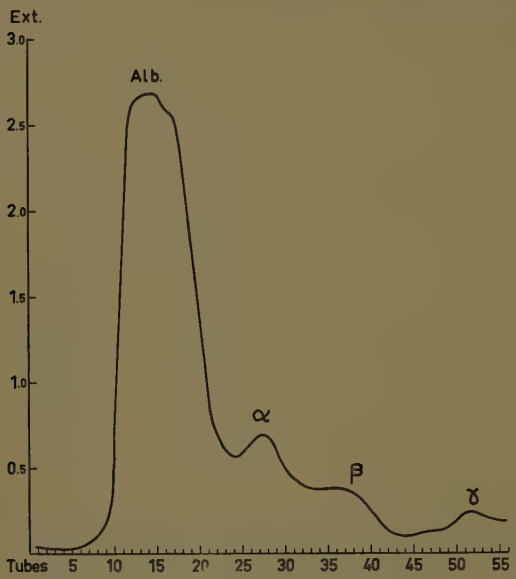


Fig. 12. Zone electrophoretic separation of pooled fetal sera.

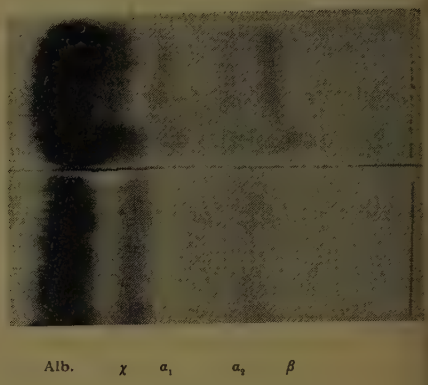


Fig. 13. Paper electrophoresis of protein fract from tube 19 of zone electrophoresis (bottom) Top: Paper electrophoresis of serum from a fetus of 19.5 cm length.

the observed period of gestation (Figs. 10 and 11).

When the electrophoresis was run at pH 7.7 (diethyl barbiturate buffer) separation of the different fractions was less satisfactory and the unknown component could not be identified.

Fetal serum was treated with half-saturated ammonium sulphate and the precipitate obtained was resolved and dialysed twice, first against physiological NaCl and then against a 10 per cent solution of macrodex. The supernatant was dialysed in the same way. After dialysis paper electrophoresis was carried out as described above. The unknown component was found in the same place in the paper strip in the supernatant but not in the precipitate.

The low protein concentration and the small amounts of serum that can be obtained from younger fetuses makes an analyses by the classic electrophoretic technique rat difficult. By pooling sera from eight fetu enough material was obtained for a z electrophoretic separation of the differ fractions. This was made at the Institute Physical Chemistry, University of Upps by courtesy of K. O. Pedersen. On the s moving side of the albumin peak a shoul was observed near the top of the cu (Fig. 12). The protein fractions of tu 18—21, *i. e.* the fractions where the unkn protein component most likely was to found, were examined by paper elec phoresis.

The unknown component in the pro fractions of tubes 18—21 was found in paper strip at the same place as before (13), but could not be identified in tubes 25 or 26. Faint traces could be identifie tube 12.

A part of the fraction from tube 20

in the ultracentrifuge and showed only ingle, slightly assymetric peak. The sedi- ntation coefficient of the unknown sub- nce could not be higher than for serum umin, possibly somewhat lower.

Pedersen (1944, 1955, 1947) has shown t fetal bovine serum contains a globulin tuin) which in the adult animal is found y in very small amounts. Fetal calf serum haracterized by large amounts of α-glo- ins and fetuin migrates with these pro- ns. It was thus of interest to compare the per electrophoretic pattern of bovine and man fetal sera. Serum samples from four f fetuses of approximately 4 to 8 months tation were examined with the paper ctrophoretic technique described above. hen strips with human and bovine fetal um were run simultaneously and compa- it was evident that in the bovine serum fraction corresponding to the unknown nponent of the human serum could be n.

DISCUSSION

It is well known that total serum protein the fetus increases during gestation. The esent investigation deals only with a rela- ely early period of fetal development and s most probably explains why a more rked change in total protein could not be nonstrated. It seems likely that the in- ase is more rapid during the later half of gnancy. Albumin increases both relatively l absolutely during the period observed —19 weeks). The relative amount of fetal umin is rather high at this period and it been shown by previous workers (Ever- k & Levens, 1950; Gleiss & Röttger, 51; Grell & Stürmer, 1953) to decrease ring the later part of pregnancy. Malmnäs (1950) did not draw definite conclusions but thought the relative amount of albumin to be constant during gestation. Grell & Stürmer (1953) also found that during the early period of fetal development serum albumin increased, but their conclusions are based on very few observations. It is not possible from the present investigation to tell exactly when the albumin curve begins to fall, but it seems likely that this does not occur at such an early date as the third-fourth month as stated by Grell & Stürmer (1953).

The increase in the absolute amounts of fetal albumin seems to follow a straight line. The calculated value at term would be 3.3 g per 100 ml, which is in very good agreement with the values given in the literature.

Most authors have found only small changes in the relative and absolute amounts of α- and β-globulins during fetal development. If the data of the present investigation are compared with normal values for the corresponding serum components at term (Oberman, Gregory, Burke, Ross & Rice, 1956; Josephson & Gyllenswärd, 1957) it seems likely that a rise in absolute values of these globulins must occur during later parts of pregnancy.

The relative amounts of α_1- and β-globulins fall significantly during the observed period and must consequently rise again during the second half of gestation as the mean values at term are higher (Oberman, Gregory, Burke, Ross & Rice, 1956; Josephson & Gyllenswärd, 1957).

Previous work has shown that the relative and absolute amount of γ-globulin increases during fetal life and that the rise is steeper after the middle of pregnancy. The present investigation is in accordance with these results.

The origin of fetal serum proteins has been much discussed and no attempt will be made here to review this problem in detail. There are at least three possibilities, a passive transfer from the maternal blood, an active production by the fetus and a placental origin.

In the present investigation a probable direct correlation between maternal and fetal albumin and α_2-globulin was found. This could possibly be explained by assuming a passive transfer from the maternal blood but can of course not be taken as evidence in favor of this conception. The inverse correlation between maternal and fetal β-globulin and the demonstration of a protein component in fetal serum not found in the mothers seem to be rather difficult to bring into accordance with a simple passive placental transfer of the serum proteins. It is also well known that the fetal serum protein pattern remains normal in spite of pronounced changes of maternal serum, *e. g.* in toxicosis of pregnancy. Furthermore it has recently been shown by Dancis, Braverman & Lind (1957) that the liver of human fetues of 3—4 months gestation is already capable of synthesizing plasma proteins except γ-globulins, and that the placenta does not significantly contribute to the proteins of the fetus.

Concerning the origin of γ-globulins, opinions are very much divided. Many authors believe that the fetal γ-globulin is derived from the mother. It is, however, difficult to see why this component should be an exception. No correlation seems to exist between the fetal and the maternal γ-globulin levels and the fact that γ-globulin synthesis could not be demonstrated in the fetal liver does not exclude a fetal production elsewhere. For the solution of this proble further investigations are necessary.

An interesting question is the nature the unknown fetal serum component. Mal näs (1950) demonstrated by the clas Tiselius technique a new component in fe sera which at pH 7.6 had a very high ra of migration, about double that of albumi The component was mostly found in se from fetuses of 4—5 months gestation. It difficult to compare these results with t present investigation as the technique us is different and not given in all details. seems, however, not very likely that the ne protein factor of the present investigation identical with that demonstrated by Mal näs. The difference in rate of migrati seems to be too great to be explained technical differences only. When the pap electrophoresis was run at pH 7.7 the on result was a very poor separation of the fra tions and no identification of the unknow component was possible. This was also t result when the electrophoresis was run pH 7.6 with a phosphate buffer.

Bang & Påby (1955) used paper electr phoresis in their study of fetal serum protei but found the separation of the fractions l satisfactory than in adults. In the young fetuses the curve obtained by photomet showed an assymetric albumin peak. Ba & Påby explained this as due to a poor paration of albumin and α_1-globulin but ha later (1956) suggested that the shoulder the albumin peak is caused by the unkno component demonstrated in the pres paper. This seems very likely. The prese of this component has also been confirm by Talavera (Personal communicati 1957).

The nature of the new protein fraction of man fetal serum is not known and the very all amounts that can be obtained make an lation and further studies difficult. It is ssible that the protein demonstrated is re- ed to fetuin of bovine fetal serum but m the results of the present investigation finite conclusions can not be drawn.

SUMMARY

The serum protein pattern of 39 human uses 9 to 19 weeks old was studied by per electrophoresis.

The relative and absolute amounts of the ferent fractions were determined and com- red with the corresponding maternal frac- ns. The results are given in diagrams.

The total protein of fetal serum probably creases during the observed period of ge- tion.

The relative amounts (per cent of total otein) of albumin, α_1-globulin and prob- ly also of γ-globulin increase whereas the nount of β-globulin decreases. No change the percentage of α_2-globulin was observed.

The absolute amounts (g per 100 ml) of umin and γ-globulin rise during the period question. No significant change in the solute amounts of α_1-globulin, α_2-globulin d β-globulin could be shown.

In fetal serum a protein component of un- own nature was demonstrated. It is situ- d between albumin and α_1-globulin in the per strip. This component, which is not nd in maternal serum or serum from pre- ture babies, constitutes a considerable part the fetal protein (average value about 10 r cent). During the observed period of station this protein fraction decreases signi- antly both relatively and absolutely. It seems likely that this protein component has a sedimentation coefficient not higher than that of albumin.

The relationship between maternal and fetal serum protein fractions is briefly discussed. It is pointed out that available evidence (including the demonstration of the unknown protein component) is not in very good agreement with the hypothesis of a passive placental transfer of serum proteins.

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TITRIMETRIC DETERMINATION OF THE NET EXCRETION OF ACID/BASE IN URINE

By K. JØRGENSEN

From the Department of Clinical Chemistry, Rigshospitalet, Copenhagen, Denmark

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Quantitative determinations of the acid/e[1] excretion in urine are valuable in cli-l cases where disturbances of the acid-e metabolism are found.

The renal mechanism of regulating the l-base equilibrium is dealt with in text ks of physiology. It shall only be recalled an excess of hydrogen ions from non-atile acids has to be excreted in the urine. re it is buffered either by a buffer system ady present, mainly phosphates, in the n of "titratable acid" (Henderson & Pal-, 1914), or by ammonia, produced by the neys. Similarly, an excess of hydroxyl s will be excreted either in the form of ratable base", or buffered by the carbon xide, always present, as bicarbonate ion. Thus the acid/base excretion in urine can expressed by the equation (Gamble, Ross Tisdall, 1923): Net acid excretion[2] =

$$r_4^+ + H^+_{titr.acid} - OH^-_{titr.base} - HCO_3^-$$

negative value of net acid excretion is numerically equal to a positive value of net base excretion. The results should be given in terms of meq/time, usually meq/24 hours.

It is noted that bicarbonate is not included in the term "titratable base". This term means (corresponding to "titratable acid") the amount of hydrogen ion required to bring a buffer system from a more alkaline pH to that of the blood. In the case of the carbonic acid-bicarbonate system, the acid form (carbonic acid) in the blood is regulated by a mechanism of its own *via* the respiratory center, so if bicarbonate should be titrated as "titratable base" this should be done at a fixed carbon dioxide tension as in blood. But this would still be wrong as any bicarbonate ion excreted in the urine represents a loss of base from the organism, even when followed by sufficient carbonic acid to give the same pH as in the blood. Imagine an organism to which nothing is supplied, and which produces no acids or bases other than the inevitable carbonic acid. The urine produced will initially have the same pH as the blood. This urine contains buffer systems which are removed from the organism with the same ratios $\frac{\text{alkaline form}}{\text{acid form}}$ as found in the blood, so the renal loss of these buffers causes no change of the blood pH, as far as

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The words "acid" and "base" are used accord-to the Brønsted-Lowry theory. An acid is a tance which yields hydrogen ions and a base; se is a substance which combines with hydro-ions to form an acid.

The term "base economy" by Gamble *et al.* been substituted by a new one as their "base" rred to cation.

other systems (*e. g.* phosphate) than the carbonic acid-bicarbonate are concerned. For this last system, the organism will only show a fall in bicarbonate, as the concentration of carbonic acid (still formed) will be kept constant by the respiratory regulation. Thereby an acidotic state is developed, due to a loss of base. In consequense of this, the organism considered, which must have a zero net acid/base excretion, must produce a urine more acid than the blood to maintain its acid-base equilibrium.

Generally, only the first two terms of the equation are determined in acid urines, and the last two terms in alkaline samples. In neutral urines, and in specimens infected with urea splitting microbes, causing an alkaline urine with considerable amount of ammonium ion, all the terms of the equation need to be determined.

During our studies on the acid-base metabolism, the following method was worked out for determination of the net acid/base excretion. It is simple, rapid and reliable, and can be used unaltered on urines with any initial pH, also if urea splitting has occured.

THE METHOD

Principle

The sample is acidified with a known amount of hydrochloric acid to convert bicarbonate to CO_2, and to dissolve any phosphate sediment. Total CO_2 is expelled by boiling. The sample is diluted to prevent later reprecipitation, and is titrated electrometrically to the pH of blood with strong base in the presence of formaldehyde.

Apparatus

pH-meter with device for acid-base titrations, or, more convenient, an apparatus for automatic titration, *e. g.* Radiometer's titrator TTT 1 with auxiliary equipment.

Reagents

1 N hydrochloric acid.

0.100 N sodium hydroxide, carbonate free.

Formaldehyde solution, 7—8 per cent, neut
Concentrated formaldehyde solution is diluted w
water, 1+4 by volume. The mixture is adjusted
pH 7.4 by sodium hydroxide. The pH is chec
daily (indicator paper), and readjusted if below

Universal indicator paper.

Procedure

Sample: 10.0 ml of the well mixed urine s
cimen (including sediment) are taken in a 150
beaker. 1 ml of 1 N hydrochloric acid (an exac
reproducible amount) is added. The mixture
heated, and kept gently boiling for ½—1 min
pH is checked with indicator paper, and if pH
found greater than 4, another portion of acid
added, and boiling repeated. 50 ml of formaldeh
solution are added. After cooling to room tem
rature titration to pH 7.40 is performed w
0.100 N sodium hydroxide, (25 ml burette).

Blind: Is run daily. 1 ml of 1 N hydrochlo
acid + 50 ml of formaldehyde solution is titra
to pH 7.40. If more than one portion of hyd
chloric acid was added to the sample, the bl
must be titrated correspondingly.

Calculation

Net acid excretion in meq/24 hours is fo
from:

$$\frac{\text{ml NaOH}_{\text{sample}} - \text{ml NaOH}_{\text{blind}}}{100} \cdot D$$

D = diuresis in ml/24 hours.

If the value becomes negative, the numerical va
can be given as a positive net base excretion.

Accuracy

The accuracy of the method can not be gi
in single terms.

If the arterial pH of the patient deviates f
7.40 a systematic error is introduced, but this
generally be considered less than 5 meq/l
"Discussion").

If the concentration of ammonium + ammo
is extremely high (greater than 500 mM) an e

ater than 5 per cent relative can be expected, this source of error can ordinarily be neglected. The main error in estimating the 24-hour ex- tion will probably be the error on the diuresis

DISCUSSION OF THE METHOD

Collection of specimen: The urine should collected with addition of some germicide *g.* tricresol, 15 drops /l), as too much ea splitting will give a final volume too ge for accurate titration. The decomposi- n per *se* will cause no error, as the NH_3 d CO_2 formed do not belong to the net id/base equation, and if some is converted NH_4^+ or HCO_3^- this will happen in equi- lent amounts, or at the expense of the rable acid/base in the sample. A possible oduction of lactic acid in sugar-containing ines must also be avoided.

The boiling with strong acid: The conver- n of bicarbonate to CO_2 with subsequent pelling of this is necessary when bicar- nate is determined by titration to a neutral d-point. Any free ammonia present will ewise bind some of the strong acid, but the value of NH_4^+ is raised correspond- gly, no error is introduced by that. A split- g of urea during the boiling has been und to be insignificantly low under the nditions given, and as any CO_2 formed ll be expelled immediately through the ge surface, no error can be caused by s step.

The dilution with formaldehyde solution: dilution before titration is a simple way to event reprecipitation of phosphates by cal- m and magnesium at the end-point. The dilution proposed has been found not to exert any significant dilution effect on the pH.

Choice of end-point pH: The end-point has here been chosen as pH = 7.40, accord- ing to the original definition of "titratable acid" (Henderson & Palmer, 1914). This, however, is an approximation made for con- venience. More correctly, the sample should be titrated to the pH of the patient's arterial blood, the pH here taken as an average value for the period of urine collection. This should be done in cases where accurate ac- counts of the acid-base metabolism are desired.[1]

This, however, is still not quite correct. In fact the end-point should be chosen so that the concentration ratios $\frac{\text{acid form}}{\text{alkaline form}}$ for the buffer systems at the end point were the same as in the organism. But this would require the unattainable knowledge about the activity coefficients in the titrated sample and in the organism for each single deter- mination.

In clinical routine determinations, how- ever, 7.40 can be used throughout as an end- point. The error, eventually introduced, will be too small to lead to false conclusions, whether the results is used to diagnose an excretory disturbance, or to follow the acid- base metabolism.

Further discussion of the end-point is given below.

[1] If, for instance, the phosphorus concentration in the urine is 100 mM, a 0.1 change of the end- point pH corresponds to a 5 mM change of the net acid/base concentration.

Table I. *Recovery of NH_4^+ by "formol-titration". Samples of 1.00 ml 1.00 M NH_4Cl (equivalent to 10.0 ml. 0.100 N NaOH) in 40 ml water (pH 5.80) are adjusted to different pH-values with 0.1 N NaOH. To each sample 10 ml of concentrated formaldehyde solution with the same pH are added. The samples are then titrated with 0.100 N NaOH to the pH values, to which they were previously adjusted.*

pH previously adjusted to	ml 0.1 N NaOH used for adjusting	pH after addition of formaldehyde	ml 0.1 N NaOH used for titration
6.00	0	5.19	1.90
6.50	0.01	5.39	6.80
7.00	0.02	5.58	9.05
7.50	0.02	5.69	9.90
8.00	0.03	5.80	9.12
8.50	1.01	5.78	8.85
9.00	2.50	5.99	7.48
9.50	5.20	6.32	4.65
10.00	7.50	6.79	2.20

Notes on the "formol-titration": The ammonium is determined with a modification of the "formol-titration" (Sørensen, 1908). A modification for direct titration of ammonium in urine was given by Bjørn-Andersen (Bjørn-Andersen & Lauritzen, 1910). He adjusted the pH of the sample to about 9—9.5 (phenolphthalein). After addition of formaldehyde, causing a change to a more acid pH, he titrated the sample back to the pH adjusted to. The amount of OH^- used was taken as equivalent to the amount of urinary ammonium (+ ammonia). His method was based on the mistaken assumption that ammonium chloride is a "neutral salt". However, it is easily seen that this method will give values of about 50 per cent of the true ones if used on acid urines, and this has also been found by experiments as seen from Table I. In acid solutions of ammonium salts only NH_4^+ occurs. If such a solution is adjusted to pH 9.3, which can be taken as the pK for the weak acid NH_4^+, half of th ammonium ion is converted to NH_3. Forma dehyde, now added, will convert the bas NH_3 into hexamethylenetetramine, whic has no acid-base properties, thus making th solution more acid. If the remaining amm nium is titrated with strong base in the pr sence of formaldehyde, the reaction will be $4\,NH_4^+ + 4\,OH^- + 6\,HCH(CH_2)_6N_4 + 10\,H_2$ *i.e.* the ammonium is now titrated as a stron acid, and can consequently be titrated to neutral or slightly alkaline reaction (9.3), desired. But only half the original amount available for this titration.

In the present method the titration star at an acid pH and is stopped at a neutral en point, and is carried out in the presence formaldehyde throughout. The recoveries a 98—99 per cent in experiments with usu amounts of ammonium, and better than per cent for concentrations as high 500 mM. The error arising from a simu taneous titration of amino acids can be ne

ted under these conditions (Sørensen 08).

The amount and concentration of formal-hyde added has been chosen arbitrarily, t has been found high enough to ensure e high recoveries just mentioned, and low ough to allow nearly constant pH at the ution.

SUMMARY

The quantity "net acid/base excretion" is fined, and briefly discussed. A method for determination, suitable for routine work, described.

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THE IMPORTANCE OF NUTRITIONAL FACTORS IN THE PATHOGENESIS OF IRON DEFICIENCY ANEMIA

By C. V. MOORE

From Department of Medicine, Washington University, St. Louis, U.S.A.

The honor of being afforded this opportunity to pay tribute to the distinguished physician, Dr. H. C. Jacobaeus, by giving this, the tenth of a series of lectures established in his name, is a great one wich I deeply appreciate. I am most grateful to Professor Hanssen, Dr. Olaf Römcke, and to Professor Waldenström, who as members of the Committee issued the invitation to me. The nine previous lecturers have been outstanding medical scientists who described in their presentations sound, exciting investigative work which must have pleased Dr. Jacobaeus' spirit if it was able to come back to the lecture halls for those occasions. The standards set by these men will be difficult for me and for future lecturers to maintain.

When one comes to Sweden and dares to talk about iron deficiency anemia or any phase of iron metabolism, he must feel particularly humble. Important contributions in this field of medicine are indelibly linked with the names of Jan Waldenström, Bo Vahlquist, C. G. Holmberg, C. B. Laurell, B. Hagberg, and others of your countrymen. Our own studies, furthermore, have not been intimately concerned with some of the newest developments in our understanding of iron metabolism: the chemical nature of storage iron, ferrokinetics, the mechanisms by which iron is transferred from cells to plasma, and the enzymatic functions of cellular iron. My associates and I have, rather, been concentrating on the problem of trying to defi the importance of nutritional factors in t pathogenesis of iron deficiency anemia.

Iron deficiency anemia is one of the mo common nutritional disorders and one of t most prevalent anemias the world ove There is a difference of opinion, howeve as to the importance of nutritional facto in the production of iron deficiency amor adults. In the United States, most worke believe that poor diet and poor absorptio alone will not cause iron deficiency in a adult unless blood loss also occurs. It argued: a) that iron is excreted in such sm amounts that a positive balance is maintain even when the diets is deficient or absorpti defective; and b) that poor nutrition leads the development of iron deficiency only wh requirements are increased by growth by chronic hemorrhage. With a deficient di or defective absorption, even the amount blood lost with normal menstruation enough to lead gradually to iron deficienc According to this concept, "idiopathic hyp chromic anemia" is an outmoded syndron On the other hand, workers in many oth parts of the world believe that when di are inadequate for long periods of tim iron deficiency develops even in adults on t basis of poor nutrition alone. In order resolve this difference of opinion, we ne to have data on the amount of iron wh is absorbed from food, and on the amount iron which is excreted by the body. Sim balance experiments to answer th questions cannot be done in a satisfact

[1] Presented as the Tenth Jacobaeus Lecture in Malmö, Sweden on October 5, 1955.

nner. Errors in the chemical determina-
n of iron are too large for the accuracy
uired, and iron is so ubiquitous that the
blems of contamination are almost in-
mountable. We have attempted to cir-
nvent some of these difficulties by using
ioactive iron. Our data are incomplete,
they permit a preliminary analysis of
problem.

My presentation will be made under four
dings:

1. A brief, orienting and schematic summary of iron metabolism.
2. Iron absorption.
3. Iron excretion.
4. An analysis of the importance of nutritional factors in the causation of iron deficiency.

A SUMMARY OF IRON METABOLISM

Iron, as an essential component of hemo-
bin, myoglobin, the cytochromes, and
er enzyme systems, is required for the
portant physiologic functions of oxygen
nsport and cellular respiration. The body
a normal, adult human contains approxi-
tely 3 to 5 grams of iron. Roughly
per cent of this total is present in the
culating hemoglobin, about 10 to 20 per
t in myoglobin, and the remainder is
red largely in the liver, spleen, kidney and
ne marrow.

The human subject absorbs iron largely,
not entirely, in its ferrous form (Moore,
bach, Minnich & Roberts, 1944). Reduc-
n of food iron from the ferric to the
rous state begins in the stomach and is
tinued in the intestine. How much iron
actually taken up by the stomach is un-
own, but absorption is probably greatest
the upper portion of the small intestine
decreases progressively in the more
tal segments of the gastro-intestinal tract.
e reason for this diminishing gradient has
er been adequately explained. It may be

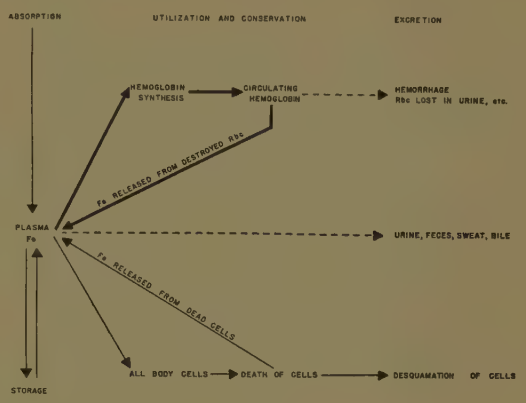


Fig. 1.

that the ileum, for instance, is not as efficient as the duodenum in absorbing iron but the difference may be due solely to the fact that insoluble, complex iron salt form by the time distal portions of the small intestine are reached. By instilling radioactive iron salts into the colon of dogs through a catheter, it has been possible to demonstrate that some small absorption may occur from the colon; under physiologic conditions, however, it is probable that very little iron is assimilated by the body from the large intestine. Iron is absorbed directly into the blood stream, rather than through the lymphatic channels (Fig. 1). When it passes into the blood plasma, it is oxidized to the ferric state and combined with a specific iron-binding globulin, a beta-1 globulin, which has been called transferrin, siderophilin or simply the iron-binding protein (Laurell, 1952). Each molecule of this specific plasma protein can combine with two atoms of iron; there is enough of it in plasma tot bind from 300 to about 420 micrograms of iron per 100 ml. The amount of iron in plasma, however, varies normally from about 50 to 180 micrograms per 100 ml; only a portion of the beta-1 globulin therefore, is bound.

The iron in plasma is transport iron, the hub of iron metabolism. From the plasma, iron may go to organs of storage, it may be utilized, or it may be excreted. A relatively

large percentage of it goes to the bone marrow where it is used for hemoglobin synthesis but some also goes to other cells of the body where it is utilized for the formation of cellular enzymes. One very important characteristic of iron metabolism is the high degree to which the body conserves the metal. For instance, when red cells are destroyed, the vast majority of the iron released from hemoglobin is re-utilized. Iron released from cells that die anywhere in the body is similarly handled and conserved. Small but significant amounts are excreted. Whenever red cells are lost, as in urine or by hemorrhage, or whenever cells are desquamated from the surface of the body or from the gastrointestinal tract, the iron which they contain must be thought of as having been excreted.

The work of Granick, Hahn, Finch and his associates, and others, has demonstrated (a) that the amount of iron stored in the tissues in a healthy adult is approximately 1 gram; (b) that the liver and spleen constitute the chief storage sites; and (c) that storage iron is found intracellularly in a protein complex as ferritin and as hemosiderin (Granick, 1951; Finch, Hegsted, Kinney, Thomas, Rath, Harkins, Finch & Fluharty, 1950). The chemical identity of hemosiderin has not been established, but the suggestion has been made that it may merely by a condensation or clustering of ferritin molecules. Ferritin has been identified as a protein, apoferritin, to which colloidal iron hydroxide-iron phosphate is bound in amounts up to 23 per cent by weight. In the liver and spleen of normal animals, there is a slight preponderance of ferritin over hemosiderin iron (Shoden, Gabrio & Finch, 1953). With increasing concentrations of tissue iron, this ratio is reversed, and at high levels the additional storage iron is deposited as hemosiderin. Of great physiologic importance is the fact that both compounds are capable of being mobilized by the body when the need for iron exists, so that they can be used for hemoglobin synthesis or other requirements.

This brief, very schematic summary some of the major characteristics of ir metabolism has emphasized how tenacious the body conserves iron. It is evident th if only small amounts of the metal are lo or excreted, the amount absorbed is of prin importance in the prevention either of ir deficiency or overload.

II. IRON ABSORPTION

A. *Technique of study and analysis of t factors which control absorption*

Iron absorption can best be studied wi test doses of radioactive iron. We ha regularly administered orally one mg iron per kilogram of body weight. One th measures both the unabsorbed radioactiv recovered in feces, and the maximum amou which appears in the circulating blood newly synthesized hemoglobin. This latt value is reached within 10 to 14 days subjects with normal or accelerated rates red blood cell formation. The results of such observations on 8 different norm subjects are charted in Fig. 2 (Dubach, C lender & Moore, 1948). The stippled porti of each column represents the per cent the test dose which was absorbed, synthesiz into hemoglobin, and found in the circulati blood. It varied from 2 to 11 per cent. T striated portion of each column represe the radioactive iron unabsorbed and

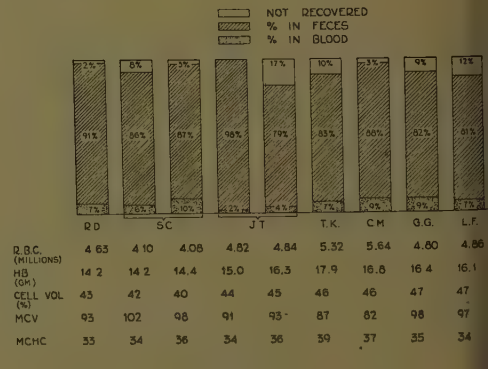


Fig. 2.

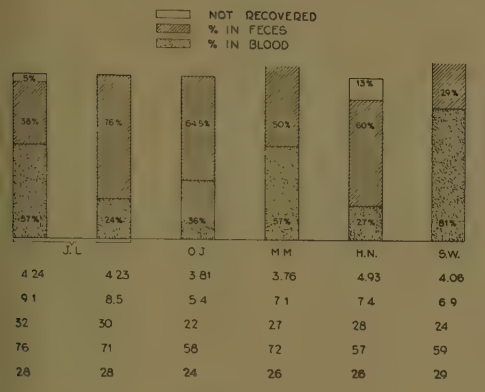


Fig. 3.

ered in the feces. The sum of these two ues usually did not equal 100 per cent; other words, a portion of the test dose s not recovered. This latter value is in- ated by the clear areas at the top of each umn. It varied from 2 to 17 per cent l results both from the error of the method l from the fact that some of the iron orbed is stored rather than used for hemo- bin synthesis.

With this technique for studying iron ab- ption, several groups of investigators have ablished two important facts: 1) human jects assimilate ferrous iron three or four es more efficiently than ferric forms; and iron deficient patients absorb inorganic ous salts much better than do normal ple. The five iron-deficient patients on om the determinations charted in Fig. 3 e obtained, for instance, had 24 to 81 per t of the administered radioactivity appear heir circulating hemoglobin. While there o doubt about this difference in absorp- between healthy and iron deficient jects, one must be careful not to overstate facts. Writers sometimes give the im- ssion that normal persons absorb no iron ll; that certainly is not true.

The mechanism by which the intestinal osa absorbs iron and helps to regulate metabolism by doing its job more effi- tly when the need is greater, is not completely understood. Granick has suggested that a protein, apoferritin, is constantly being formed and broken down in the mucosal cells of the intestinal tract; according to this hypothesis, the degradation of apoferritin stops when the apoferritin combines with iron to form ferritin (Granick, 1951). The presence of ferritin is thought to be associated with a block of further absorption until the ferritin can give up its iron to the blood stream. In support of this concept, is the fact that the uptake of radioactive iron can be blocked by the prior feeding of a fairly large dose of inert iron. To demonstrate this effect, Stewart, Yuile, and their colleagues gave 100 mg of inert iron to chronically anemic and iron deficient dogs (1948). They then waited at least five hours before giving the test dose of radioiron, in order to avoid mixture of the two doses in the stomach. The blocking effect was maximum at the end of five hours, when only 33 per cent as much iron was absorbed as during the control period. The blocking effect gradually decreased as the interval between inert iron and radioiron was lengthened, and had disappeared after about 24 hours. In addition, Granick fed massive doses of iron to guinea pigs and noted accumulations of ferritin in the mucosa of the entire gastrointestinal tract. He postulated that "mucosa block" occurs when the mucosal cells become saturated with ferrous ions and that these ferrous ions are in equilibrium, in turn, with the ferric iron of the blood plasma and with the ferritin stored in the mucosa. Endicott and his associates (1948), however, were unable to confirm these observations in a study of histochemical accumulations of iron after feeding the metal to guinea pigs, and concluded that the visible granular iron in the duodenal epithelium exerted no demonstrable effect on the amount of iron absorbed and did not appear to be a morphologic expression of the mucosal block.

The relationship of ferritin to the process of iron absorption has, therefore, been questioned. But even if we accept the role

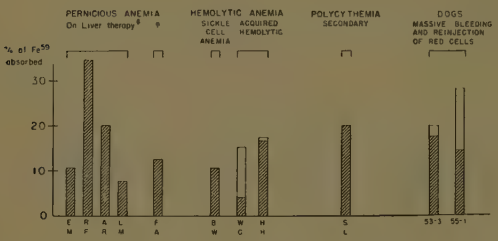


Fig. 4.

* Liver therapy had been begun 3 to 4 days before the iron absorption study † Spontaneous remission
Radiactive iron (1 mg per kg) was given by mouth as $FeCl_2$ or $FeSO_4$ (reduced with ascorbic acid)
////// per cent Fe^{59} in blood □ per cent Fe^{59} retained but not found in red cells

of ferritin, we must still explain why iron deficient subjects give up the iron from the ferritin to the plasma so much more rapidly than do normal individuals as to increase absorption. At first it was thought that the low plasma iron found in hypochromic anemia might lead ferritin to give up its iron more rapidly, but recent work has shown that absorption is independent of the plasma iron level. The level of iron stored in the body is also not the determining factor since some patients with hemochromatosis, untreated pernicious anemia and hemolytic anemia assimilate iron quite well (Stewart *et al.*, 1950; Bothwell, van Doorn-Wittkampf, DuPreez & Alper, 1952). Some of these results are illustrated in Fig. 4 along wi data on one patient with secondary pol cythemia. Illustrated also are data on tw dogs which were made anemic by massi bleeding and then reinjected several da later with their own red cells to correct t anemia that had been produced. In the inte val between the hemorrhage and restorati of their red cell mass, erythropoiesis h been stimulated. When a dose of radioir was then given, absorption was significant greater than it had been during a contr period. In many instances, accelerated ra of erythropoiesis are associated with greate than-normal iron absorption.

Granick next suggested that anemia, wi its attendant low oxygen carrying powe might lower the oxygen available to t mucosa, affect the relative redox level the cell, and thereby accelerate the transf of iron from ferritin in the mucosal cells the plasma so as to increase the absorpti capacity of the mucosal cell. At first glan it does appear as if anemia *per se* m be an important factor. Pyridoxine-deficie dogs, anemic but rich in stored iron, abso the metal well. Patients with hemoly anemia and dogs with phenyl hydrazi induced anemia have no difficulty absorbi iron. Two observations, however, cast dou

Table I. *Absorption of radioiron (ferrous sulfate) by five women with iron deficien anemia before and immediately after series of transfusions — No iron therapy given.*

Pt.	Before Transfusions					After Transfusions			
	Rbc. 106/cu. mm.	Hb. g.	C.V. per cent	Serum iron µg/100 ml	Per cent Fe^{59} absorbed*	Rbc. 106/cu. mm.	Hb. g.	C.V. per cent	Per cen Fe^{59} absorbe
T.F.	4.10	5.6	23.5	17	26.2	5.44	12.0	41	17.8
M.F.	2.30	4.5	17.5	25	40.7	5.03	12.3	40	36.3
E.H.	3.44	4.1	21	4	47.3	5.05	13.8	40	10.0
H.M.	4.26	9.8	33	21	29.0	5.65	14.0	45	7.5
H.P.	4.15	6.6	25	22	31.3	4.98	11.9	37	26.8

*) Radioiron which appeared in circulating hemoglobin used as the measure of iron absorbed. Test d provided 1 mg. Fe / kg. body weight.

this suggested effect of available oxygen: we have shown that iron-depleted dogs orb iron equally well under atmospheric ditions and while maintained in a cham- containing 70 per cent oxygen; 2) iron orption was measured in patients with n deficiency anemia before and after their noglobin levels were raised to normal hin a few days by transfusion. No iron rapy was given. The amounts of iron ab- bed in 3 of 5 experiments were similar able I).

The above discussion may appear labored, it is important to point out how little actually know about the mechanims which trol the absorption of iron. The only eralizations we can make are these: (a) n-deficient subjects do absorb iron more ciently than do normal individuals; and whenever red cell formation is stimul- d, as in the hemolytic anemias or after norrhage, the absorption of iron seems be greater than normal. But exactly how greater absorption is brought about ains a mystery. The level of plasma iron, amount stored in tissues or the degree anemia seem to have been eliminated as trolling factors. Even the role of ferritin st be regarded as suspect.

B. *Absorption from foods*

Most of the information about iron absorp- n is of little nutritional significance since nas been obtained from experiments in ich inorganic iron salts were fed to ients or animals. The ionizable iron in ny foods has been measured on the umption that only this portion is available absorption, but when balance studies are empted to determine the amount retained, technical difficulties become enormous. cently, foods containing radioactive iron e been obtained by growing vegetables in rient solutions to which the isotope was ed or by injecting iron into hens so that s, liver and muscle contained radioiron oore & Dubach, 1951). These foods n were cooked or prepared as they would

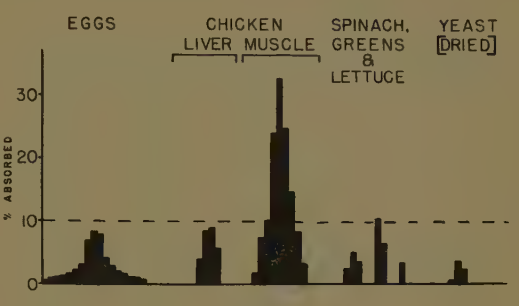


Fig. 5.

be in a normal diet and were fed to fasting subjects. Absorption was measured in the manner already described. Fig. 5 presents the results of a number of such observations. The per cent of iron absorbed is indicated along the vertical scale. Each column represents a separate experiment. A horizontal, dotted line is drawn at the 10 per cent level to emphasize the fact that with very few exceptions, the most striking being from chicken muscle, less than 10 per cent was absorbed by normal persons. When similar observations were made on iron deficient patients very few of them absorbed iron from eggs any more efficiently than did normal persons. In a few observations made so far with chicken liver, spinach, and dried brewers' yeast, it appeared as if patients with iron deficiency anemia do tend to absorb these forms of food iron somewhat more efficiently (Fig. 6). When other foods were

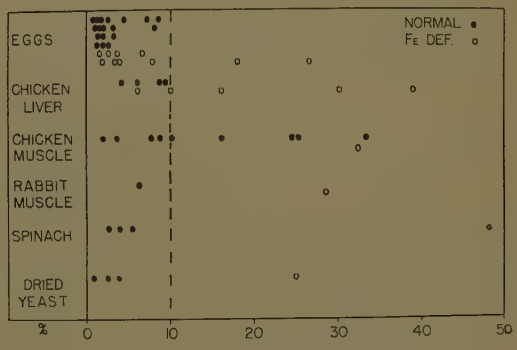


Fig. 6.

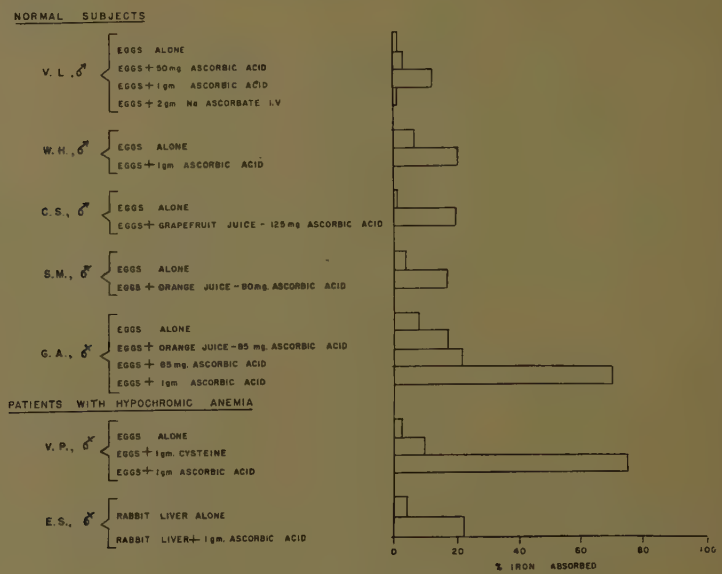


Fig. 7.

added to eggs, the only ones which had an augmenting effect on assimilation of the radioiron were citrus fruit juices which contained a relatively high concentration of ascorbic acid (Fig. 7). As a matter of fact the increased absorption produced by 200 ml of orange juice, and the increase produced by a comparable amount of crystalline ascorbic acid were similar (see G. A., Fig. 7). The addition of 0.25 to 1 gram of crystalline ascorbic acid to a number of different food substances increased absorption in every case, having perhaps a slightly greater effect in the iron deficient subjects (Fig. 8). It is suggested that ascorbic acid produces this result by bringing about a more efficient reduction of ferric iron in food to the ferrous state.

A preliminary attempt has also been made to evaluate the importance of gastric hydrochloric acid in the absorption of iron from food. For many years it has been taught that patients with hypochlorhydria or achlorhydria probably assimilate iron poorly. In such patients, however, we were not able to increase the absorption of iron by addi 60 ml of tenth normal hydrochloric acid the food, or by adding enough one norm hydrochloric acid to reduce the pH of t mixture to 1.5 before it was given stomach tube. On the other hand, 250- 1000 mg of ascorbic acid did increase a sorption very significantly even though had comparatively little effect on gast acidity.

These results can be summarized in t following manner.

1. Radioiron has been incorporated i eggs, chicken muscle, chicken liver, musta greens, spinach, and brewer's yeast.

2. With three exceptions, the absorpti of radioactive iron from foods by forty n mal subjects has been less than 20 per c with only six exceptions it was less th 10 per cent.

3. Ascorbic acid, and foods containi ascorbic acid, have enhanced the absorpti of food iron.

4. Iron deficient subjects have absorb iron from foods somewhat better than

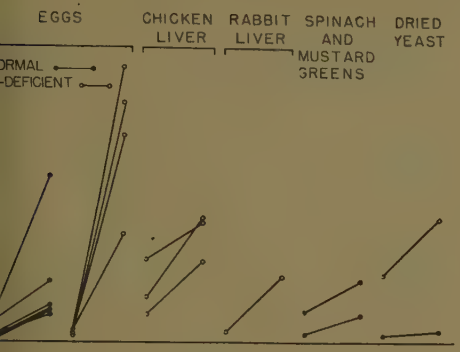


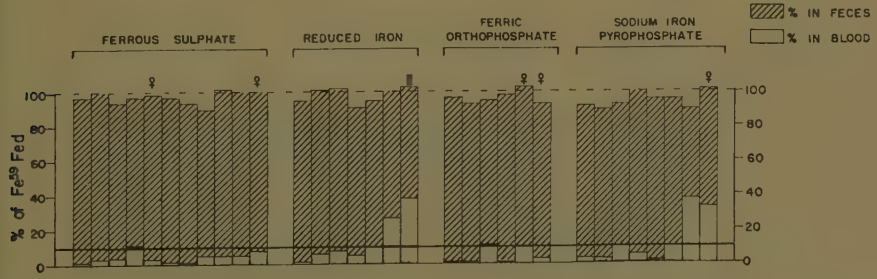
Fig. 8.

nal subjects, particularly if the food iron reduced by ascorbic acid.

In preliminary observations, addition cid when subjects had hypochlorhydria not influenced absorption of food iron.

he diet of healthy adults in the United es contains approximately 12 to 15 mg on per day. If the above figures are used basis for calculation, the amount of iron rbed from food per day probably varies about 0.6 to 1.5 mg. Poor diet, infec- steatorrhea, or diarrhea would decrease amount even further. The effect of orrhea in reducing the absorption of iron patients with hypochromic anemia has ntly been very beautifully demonstrated Badenoch & Callender (1954) in Eng-

Of related interest are observations recently completed on the absorption of iron from bread baked with flour which had been enriched with radioactive iron (Steinkamp, Dubach & Moore, 1955). In the food fortification program in the United States, iron is added as reduced iron, as sodium ferric pyrophosphate, as ferric orthophosphate, or as ferrous sulfate in amounts sufficient to provide 6 to 8 mg of iron per pound loaf of bread. There has been, however, no adequate evaluation of the effectiveness of this particular type of fortification, and several of the preparations are so insoluble that people have wondered how valuable they are. Each of these four iron preparations was obtained in radioactive form and baked into bread along with other substances used in fortification by a pilot bakery. The baking process similated as closely as possible that used by large commercial bakeries. Four slices of the bread were then fed to healthy subjects and absorption measured. Twenty-eight of the 32 subjects absorbed and utilized between 1 and 12 per cent of the iron, regardless of the form of iron added. The other 4 persons assimilated from 26 to 38 per cent of the radioiron, but there was reason to suspect that their iron stores were suboptimal in each instance (Fig. 9). Apparently, the various iron preparations are changed in the baking process, probably to a complex salt, so that all are about equally



THE SUBJECTS WERE HEALTHY MEDICAL STUDENTS. EACH ATE 4 SLICES OF BREAD ENRICHED WITH ONE OF THE RADIOACTIVE IRON PREPARATIONS

Fig. 9.

effective. The only three patients with iron deficiency anemia included in the study assimilated 45 to 64 per cent of the iron taken. Addition of 1 gram of ascorbic acid to the bread eaten by six of the healthy subjects increased the absorption of iron two to three times.

III. EXRETION OF IRON

Because conservation of iron is so tenacious and because the amount excreted is so small, many people have mistakenly assumed that not iron whatever is lost from the body except as shed blood. The error of this assumption becomes obvious from the following considerations. All cells in the body contain iron. When leucocytes and epithelial cells are discharged in body secretions, when erythrocytes appear in urine, when cells are desquamated from the skin or the mucosa of the intestinal tract, and even when hair grows, some iron is lost. Attempts to estimate this amount and to determine in additon how much is excreted in other ways has proved a very difficult task. The metal is so ubiquitous that it has been almost impossible to differentiate between excreted iron and that present because of contamination or because it was not absorbed. Radioiron has provided a partial, but not a complete solution to the problem.

The isotope has been injected intravenously in tracer amounts into normal subjects and patients with hypochromic or hemoly anemias (Dubach, Moore & Callend 1955). Five day fecal collections have th been made at intervals up to 150 days; t long period was used because most of injected radioiron was promptly synthesi into hemoglobin and we wanted the coll tion periods to be greater than the 120 lifespan of the erythrocytes. Every fe specimen on every subject contained detectable amount of radioactivity; amount averaged 0.01 per cent of the d per day for normal subjects (Fig. 10.) Th iron-deficient patients excreted much smal amounts while one young woman with hemolytic (sickle cell) anemia excreted mo No significant increase in fecal radioactiv was detected at or near the 120th day af the injection had been given. It is diffic however, to calculate from these figures w the fecal excretion of all body iron — in as well as radioactive — would be. T calculations were made: one from the ra of total hemoglobin iron to radioactive hem globin iron; the other from the ratio estimated total body iron to the injec dose of the isotope (Fig. 11). Fecal excret calculated in these ways for normal subje varied from 0.3 to 0.5 mg per day. For ir deficient patients the value was about o tenth as much. While these calculated val are admittedly estimations, one can reasonably certain that they do not v from true figures by more than 100 per c

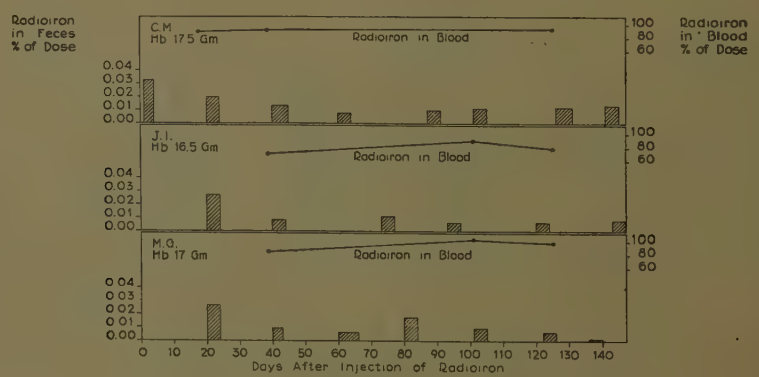


Fig. 10.

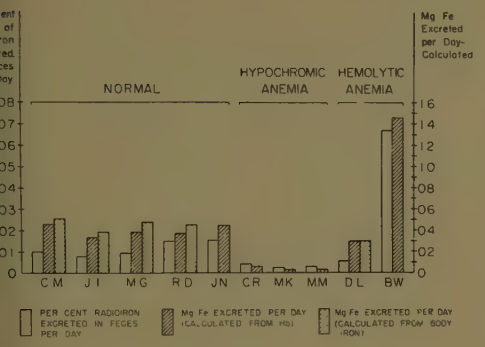

Mg Fe Excreted per Day-Calculated
NORMAL
HYPOCHROMIC ANEMIA
HEMOLYTIC ANEMIA
CM JI MG RD JN CR MK MM DL BW
PER CENT RADIOIRON EXCRETED IN FECES PER DAY
Mg Fe EXCRETED PER DAY (CALCULATED FROM Hb)
Mg Fe EXCRETED PER DAY (CALCULATED FROM BODY IRON)

Fig. 11.

e cannot differentiate with this method ong the following three possible sources fecal iron: a) true excretion, b) des-mated mucosal cells, and c) iron deli-ed to the duodenum via the bowel and not pletely absorbed.

ome of the injected radioiron was also nd regularly in sweat for at least 320 s, the longest time after administration a collection was made. The amount was ll, but definitely measurable. We were able to determine whether it came from at glands by a process of true excretion rom desquamated epithelial cells. Calcula-s indicated that under normal conditions total iron lost from dermal surfaces is ainly less than 1 mg per day and probably more than 0.5 mg. It is of interest that ioactivity could also be detected in hair pings obtained several months after the ction of radioiron and washed with dilute l to free them from surface (sweat) con-ination.

To the above values must be added the ll amount of iron found in urine. From e figures, one can estimate that the adult e loses or excretes between 0.5 and mg of iron per day; the median value mg is probably not in error by more n 20 to 25 per cent. Menstrual blood flow 35 to 70 ml every 28 days in a normal nan with a hemoglobin value of 12 grams ld account for an additional average loss .5 to 1 mg per day.

IV. IMPORTANCE OF NUTRITION IN THE PATHOGENESIS OF IRON DEFICIENCY ANEMIAS

Even though the data presented for the absorption of iron from food and for the excretion of iron are admittedly incomplete and provide approximations only, they do permit a better evaluation of the importance of nutritional factors in the pathogenesis of iron deficiency than has previously been possible. If the adult male absorbs an average of 10 per cent of the iron in a diet that contains 12 to 15 mg per day, he retains 1.2 to 1.5 mg. Since he excretes only about 1 mg or less of iron per day, he maintains a positive balance rather easily. The adult woman, however, during the years of menstruation and child bearing tends to eat less and loses additional amounts. A mother furnishes her fetus during gestation with about 300 to 500 mg of iron, or between 1 and 2 mg per day during the duration of her pregnancy. The volume of menstrual blood is normally approximately 35 to 70 ml. If the hemoglobin value of that blood is 12 grams per 100 ml, then 14 to 28 mg of iron would be involved. Spread evenly over a 28 day menstrual cycle, this equals 0.5 to 1 mg per day. Iron balance in a young woman, therefore, is precarious so that poor diet or poor absorption may lead to iron deficiency even though menstrual loss remains normal. Frequent pregnancies or any increase in menstrual flow make her all the more susceptible. Women are often unaware, furthermore, of the fact that they might be losing several times the normal amount of menstrual blood. Menstrual pads are so efficient at absorbing blood that false impressions are obtained. The pads in Fig. 12 were prepared by injecting onto their surfaces 5, 10, 15, and 20 ml of blood, respectively. The four at the top were not disturbed. Those at the bottom were rubbed together at the edges to simulate the agitation which might occur with walking. In no instance did the blood soak through more

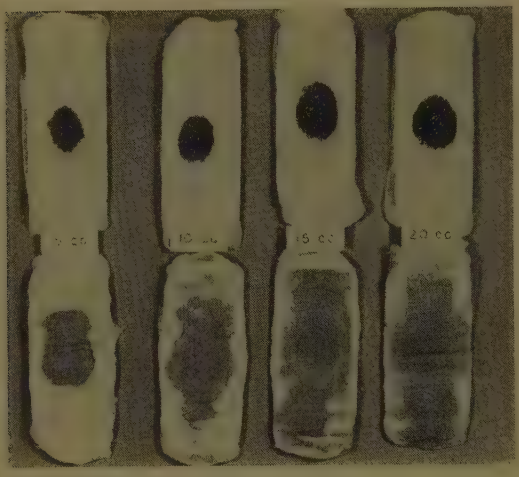


Fig. 12.

than three fourths of the material. Women physicians who inspected and watched the preparaion all agreed that at least the pads to which 10 ml had been added would be considered "normal". It is evident that a woman who uses 10 to 15 pads per period might easily lose more than 100 ml of blo without being aware of any abnormalit I have seen two intelligent, medically train women with hypochromic anemia who extracted pads contained in excess of 200 of blood per period; neither one had be aware that her blood loss was unusual.

Almost nothing is known about iron excr tion in children, but the infant and the gro ing child need iron to increase their blo volume, their myoglobin volume and t respiratory enzymes required by all cel Since the body of an infant contains abo 0.5 gram and that of an adult 3 to 5 gra of iron, there must be a net gain during t first twenty years of life of 2.5 to 4.5 gram this net gain averages 0.12 to 0.22 gra per year or about 0.35 to 0.6 mg per da In all probability, therefore, the positive ir balance maintained by normal childr during their most active growth must slight so that poor diet or poor absorpti could readily produce iron deficiency.

It is much more difficult however for ir deficiency to develop in the adult male

Table II. *Calculation of time required for development of iron deficiency on nu tritional grounds alone in hypothetical patients* (see text).

	Adult male Hb. 15 G./100 ml. Bl. vol. 5000 ml.		Post-menopausal woman Hb. 14 G./100 ml. Bl. vol. 4000 ml.	
A. When normal				
Total Hb. iron	2500 mg.		1900 mg.	
Storage iron	1000 mg.		500 mg.	
Total		3500 mg.		2400 mg.
B. After development of Fe deficiency (Hb. of 7.5 Gm./100 ml.)				
Total Hb. iron	1250 mg.		950 mg.	
Storage iron	0 mg.		0 mg.	
Total	1250 mg.		950 mg.	
Deficit in Hb. and storage iron (with this degree of hypochromic anemia)		2250 mg.		1450 mg.
Time required to produce deficiency if no iron is absorbed and 1 mg. excreted per day		2250 days (6.3 yrs.)		1450 days (4 yrs.)

post-menopausal woman on a purely tional basis without any associated blood The calculations in Table II, based on hypothetical patients, demonstrate that ese two persons excreted 1 mg of iron lay and absorbed none at all, six and years, respectively, would be required e they would become deficient enough ave only 7.5 grams of hemoglobin per ml. These figures ignore the evidence as patients become iron-deficient they ete smaller amounts of the metal. Any contained in the deficient diet, further-, would tend to be assimilated with er than normal efficiency unless there a serious absorptive defect in the tinal tract. Both factors would consider- delay the appearance of iron deficiency ia. One is forced to admit, however, heoretical possibility that men or post- opausal women who consume very de- t diets or who have absorptive defects l over a period of many years develop deficiency on a nutritional basis alone. he other hand, it seems far more likely patients with inadequate diets or poor rption also lose small amounts of blood go undetected. The bleeding may be t and intermittent so that diligent search quired for its detection. There is some ence that blood added daily in small unts to the stomach may be so completely sted that tests for occult blood in the remain negative (Mendeloff, 1953). hemorrhage, even though small in unt, inadequate iron intake or absorption becomes of major importance in the ogenesis of hypochromic anemia.

SUMMARY

utritional factors are of major impor- e in the production or prevention of iron iency anemia. Healthy persons probably tain a positive iron balance by a nar- r margin than was formerly believed. roximately 5 to 10 per cent of food iron s to be assimilated by normal adults; daily retention on a diet containing 12 to 15 mg of iron, therefore, may be estimated to be about 0.6 to 1.5 mg. The amount of iron lost from the body each day in all ways except as blood seems to be between 0.5 and 1 mg. The added requirements of children and young women to compensate for growth needs and menstrual flow place them in a precarious state of iron balance so that poor diet or poor absorption can readily lead to the production of hypochromic anemia. In adult men or postmenopausal women, however, nutritional factors appear to be of less importance in the pathogenesis of iron-deficiency. If purely nutritional iron deficiency ever occurs in these people, many years would be required for its production. It is more likely that occult, intermittent bleeding, often difficult to detect, must also be present along with inadequate diet or malabsorption before severe degrees of iron deficiency develop.

ACKNOWLEDGEMENT

Dr. Reubenia Dubach has been a constant associate in the studies described in this presentation, while Dr. Sheila Callender, Dr. Ruth Steinkamp, and Dr. Elmer Brown have participated as coworkers in various portions of the work.

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Technique and Practical Problems

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NEW TIME-SAVING APPARATUS FOR CUTTING PAPER-TRIPS FOR ELUTION IN PAPER ELECTROPHORESIS

By E. WESTENGAARD

From the Department of Surgery C, Rigshospitalet, Copenhagen

(Received for publication March 18, 1957)

n preparing the electrophoretic diagram aper electrophoresis we can either per- n direct photometry or cut the paper into os, elute and then determine the extinc- coefficient in various tubes. If the paper ut with intervals of the same size, the racteristic electrophoresis curve can be le, the extinction coefficients being plot- along the ordinate in accord with the ective, equally large strips in the entire th of the electrophoresis paper along the issa.

Most authors simply state that the paper uld be cut into strips according to the pose, either corresponding to the fractions aikel, 1955; Laurell, 1956) or with de- e intervals (Kunkel & Tiselius, 1951), these procedures are time-wasting. ffiths (1953) prefers direct photometry in er to avoid the troublesome cutting and numerous readings. Harboe uses a brass e with a number of transverse slits with rvals of equal size according to the pur- e. After drying, the electrophoresis paper laced on the plate and cut into strips with azor blade passing into the slits. The er has previously used a photographer's mming board, where the paper has to be ned forward for cutting into equally large os. However, even the slightest inaccuracy ses the color content to be either too high oo low in the test-tube concerned at the ense of the neighboring tubes. The curve becomes incorrectly drawn.

After construction of the apparatus [1] described below it has, however, become possible to cut the paper into strips for elution much more easily, more rapidly and more accurately.

PROCEDURE

A number of razor blades, measuring 0.1 mm in thickness, separated by means of perforated steel plates, are clamped by two traversing steel bars. The dried electrophoresis paper is placed on the razor blades, which project a little more than 4 mm with equally large intervals, here exactly 3 mm (Fig. 1). A piece of fairly stiff cardboard is placed on top of the paper, and the whole is placed upside down in an ordinary hand-press. The hand-press can exert a uniform pressure on the vertical, very thin razor blades, resulting in a uniform, accurate and fine cutting without spoiling the razor blades. The steel block is removed and turned. The cardboard, which is fixed by the razor blades, is removed. The separated paper strips are stuck between the edges of the razor blades. One by one the strips are transferred with a pair of tweezers to a number of test-tubes, in which elution may then take place directly (Fig. 2).

The whole procedure only takes about 1 minute in spite of the greater accuracy obtained, and the apparatus can be used routinely by anybody.

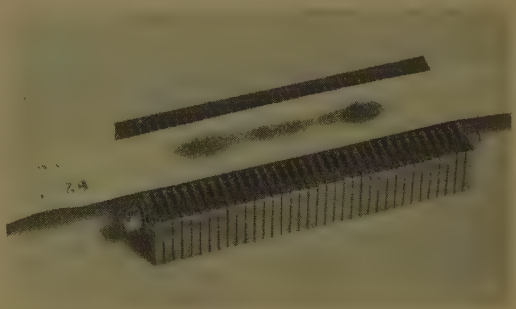

Fig. 1.

[1] Made by Dansk Laboratorieudstyr, Ryesgade 3, enhagen.

Fig. 2.

A cheaper and lighter material, such as plexiglass, might be used with advantage. It appeared that the razor blades remained sharp for a long time when thus used; they can, however, be easily renewed when necessary.

SUMMARY

A new time-saving apparatus for cutti paper-strips for elution in paper electroph resis is described. It is fitted with raz blades, placed at equally large intervals means of steel plates. The electrophore paper can be separated by means of a han press into uniform, small strips, which a then eluted.

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DEVICE FOR AUTOMATIC ANALYSIS OF CARBON ONOXIDE IN GAS SAMPLES FOR DETERMINATION OF LOOD VOLUME ACCORDING TO SJÖSTRAND'S METHOD

By H. LINDERHOLM and B. SÖDERSTRÖM

From The Laboratory of Clinical Physiology, The Caroline Hospital, Stockholm, Sweden.

(Received for publication April 26, 1957)

The CO method for the determination of total amount of hemoglobin and of the od volume according to the rebreathing nique described by Sjöstrand (1948), re-res a great number of analyses of CO in piratory gas samples when the method is d on a large scale. An arrangement for omatic analysis of CO in the gas samples been developed in order to meet an in-ased demand for blood volume determina-s as well as for the determination of en-enous CO-formation (Sjöstrand, 1949) pulmonary diffusing capacity with CO. short description of this arrangement is en.

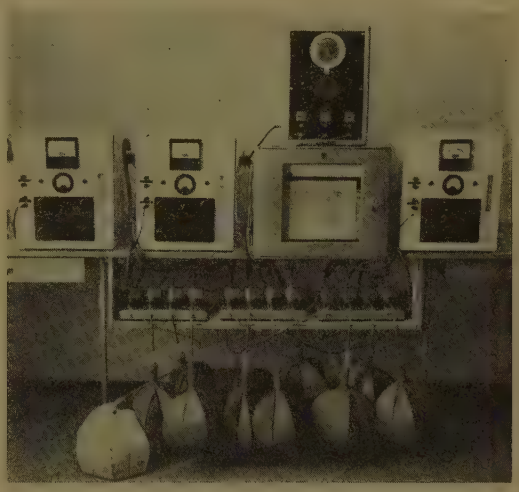
METHODS

Device for automatic CO analysis.

The device for automatic analysis of CO was structed to perform analyses from about 15 rminations of blood volume, *i. e.* 45 CO ana-s, per day. It consists of 1) three CO meters, a multipoint millivolt potentiometer recorder, our electromagnetic three way valves for each meter, and 4) a specially constructed timer. set-up is shown by the fig.

CO meter and recorder

CO meters (type SL 2, AB Stålex, Stockholm) he hopcalite type were used as described by derholm & Sjöstrand (1956). The operating ciple is briefly as follows: CO free air is conti-isly sucked through the CO meter at a constant of about 1.5 l/min. If a gas sample containing is sucked into the CO meter the temperature ge produced by the catalytic combustion of CO easured electrically and recorded by the poten-eter recorder (Philips, type PR-3200 A/00). deflection of the recorder over a wide range roportional to the amount of CO, provided the sample is introduced for less than 20 seconds. r of the 12 channels of the recorder were used each CO meter.



Electromagnetic three way valves.

The CO-free air passes four electromagnetic three way valves (Asco, type 83143) before it enters the CO-meter. A rubber bag with a gas sample to be analysed may be attached to each of the three way valves. When a valve opens gas is sucked into the CO meter from the bag. When it closes CO-free air again passes into the CO meter.

Timing device

The timer was constructed in co-operation with AB Reflex, Stockholm. It consists of Reflex clock (Swedish patent Nos. 129398 and 148750, AB Reflex, Stockholm) which during an analysis program of one hour made a three way valve open in a preset sequence every second minute, in such a way that a valve opened every 6th minute for each CO meter. The three way valves were kept open for 10 seconds by means of another timing clock and during that period about 250 ml gas were sucked into the CO meter. The 10 second period was correct within ± 0.05 seconds.[1] During a one hour

[1] This was measured by recording the pressure change at the three way valve with a strain gauge manometer and an electrocardiograph.

program of analyses each valve opened twice and double analyses were thus obtained for each gas sample. One of the gas samples, attached to the first of the four three way valves of each CO meter, was a standard gas of known CO concentration. When a one hour program was finished another program started automatically performing analyses of the standard gas every 12th minute. This program continued until, after exchanging the bags, a new analysis program was started.

RESULTS AND CONCLUSIONS

In order to get correct results it is essential to have a constant rate of air flow through the CO meters. Also the 10 second period, during which the three way valves are kept open, must be exact and constant. With our arrangement the error of a single determination of 0.01—0.02 per cent CO in air is 1.5—2.0 per cent of the mean value (coefficient of variation), while with a gas concentration ten times lower the error was about 3 per cent.

The automatic device is time saving a 50 duplicate gas analyses each day are easi performed. Measurement of the record curves may be performed at a convenie time. The arrangement may be adapted f use in connection with other physical metho of gas analysis.

ACKNOWLEDGEMENTS

This work was supported by grants from "Fo sam's rehabilitation fund" and the "Swedish R search Council".

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Letters to the Editors

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UBE ANALYSIS, UROPEPSIN EXCRETION AND AZURE A METHODS IN ESTIMATION OF GASTRIC SECRETION

The many drawbacks of the classical tube lysis technique for the determination of tric secretion have been generally recog-ed. In the search for tubeless procedures ortant advances have been made by Segal, ler & Morton (1950) and Segal, Miller Plumb (1955) in introducing indicator- exchange compounds, which dissociate at pH values low enough to indicate presence of free hydrochloric acid in the tric content. The originally used chinin-n complex has recently been replaced by azure A-resin compound, which is much re practical because the dye released from exchanger is easily demonstrated in urine its blue color. Comparative studies with standard gastric intubation method pub-ed so far have indicated a high rate of ıracy (97 to 100 per cent) for the azure rocedure in distinguishing achlorhydrics n acid secretors (Segal *et al.*, 1955; Segal Miller, 1955; Galambos & Kirsner, 1955; dbloom, Feinstein & Eiber, 1955; Bolt, ius & Pollard, 1957). In the present pre-nary paper the results of tube analysis azure A excretion after insulin stimula-, and of basal uropepsin excretion are pared with each other on the same indi-ıals.

MATERIAL AND METHOD

ll patients included in the study were hospital-The following sequence of investigations was rmed in each. On the first morning at 8.00 gastric intubation was made. If a Congo paper indicated the presence of free HCl the tube withdrawn at once. In case the Congo test negative, 16 units of regular insulin were given intravenously to the subject and samples of gastric content were drawn by suction at 20 minutes' intervals until Congo paper turned blue, showing the presence of free acid. If the fourth sample (at 80 minutes after insulin) was still negative the tube was removed and the subject was reintubated three hours after dinner (at about 8 p.m.). When the Congo test still indicated the absence of acid the result of the tube analysis was held to be negative. During the following night, 12-hour urine was collected for the assay of basal uropepsin excretion according to the method of West, Ellis & Scott (1952). On the second morning the patient voided at 7.30 a.m. and the urine was discarded. Immediately thereafter 300 ml of water and at 8.00 a.m. 16 units of regular insulin intravenously were given. Thirty minutes later the subject voided again and this urine was used as a control sample. Then 2 gm of azure A-Amberlite XE-96 compound[1] prepared exactly according to the procedure given by Segal *et al.* (1955) were administrated by mouth with one glass of water. Urine was collected for a 2-hour period after ingestion of the granules and the excretion of azure A during this time was determined quantitatively with Beckman B spectrophotometer at 620 mμ. Before the reading each sample was diluted to 300 ml with water and boiled in water bath for 10 minutes. The urine taken at 8.30 was treated in the same manner and used as a blank. Excretion of more than 0.3 mg of azure A per two hours was regarded as indicative of the presence of free acid.

RESULTS AND DISCUSSION

Altogether 60 individuals have been tested so far. Of these the tube analysis showed the presence of acid in 38 cases. Consistent results by the other two methods (uropepsin excretion over 10 units/hour and azure excretion over 0.3 mg/2 hours) were obtained

[1] The authors are grateful to A. Ilmanen & Co., Helsinki, for kindly providing the resin.

in 32 cases. In the remaining 6 cases the azure test was negative in 3 cases with an uropepsin excretion over 10 μ/hour in 2 cases with uropepsin below this limit. However, 4 of these azure negative cases showed signs of an impaired renal function (hematuria and/or reduced PSP-clearance) and one had diarrhea at the time the test was made. In one case both the tube and the azure tests showed acid with an uropepsin value of 6 μ/hour.

In the tube analysis 22 individuals were achlorhydrics. Consistently negative results by the other two methods were obtained in 20 of these subjects. In 2 cases the azure test was positive with a negative Congo test, one of these having also an uropepsin excretion over 10 μ/hour.

Thus, if the cases with a known interfering factor are excluded there remain 2 cases where the results of azure and tube tests were inconsistent and 2 cases where the results of uropepsin assay and tube test were inconsistent. No correlation was evident between the levels of azure and uropepsin excretions, a result highly expectable on the basis of the earlier failures to correlate dye excretion and the quantity of acid secretion (Galambos & Kirsner, 1955).

The material investigated is for the present too small to need more statistical treatment. However, it is seen that, in agreement with the previous experience referred to above, the azure-resin principle gives consistent results with the direct analysis of gastric content in a percentage high enough to warrant its routine use for the detecti of achlorhydria. The accuracy can be furth increased, without much additional work, combining it with the determination of bas uropepsin excretion. Only in cases whe the results of these two tubeless methods a contradictory the gastric intubation is n cessary. In patients with impaired ren function or decreased intestinal resorpti (diarrhea, pyloric obstruction) the result the azure test is reliable only if positive.

Department of Medicine,
Malmi Hospital,
Helsinki, Finland.

June 1957.

E. Halminen,
E. A. Nikkilä.

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DETERMINATION OF ORNITHINE CARBAMYL RANSFERASE WITH MICRODIFFUSION TECHNIQUE

n a recent communication a method for determination of ornithine carbamyl nsferase (OCT) in serum was described. e level of this enzyme activity seems to be cifically elevated during liver and gall eases (Reichard, 1957).

During the OCT reaction citrulline is for- l from ornithine and carbamyl phosphate. highly purified enzyme (Reichard, 1957) shown to be identical with the enzyme cribed by Krebs, Eggleston & Knivett 55) which catalyzed the arsenolysis of ulline according to:

$$\text{Citrulline} \xrightarrow{\text{arsenate}} CO_2 + NH_3 + \text{ornithine}$$

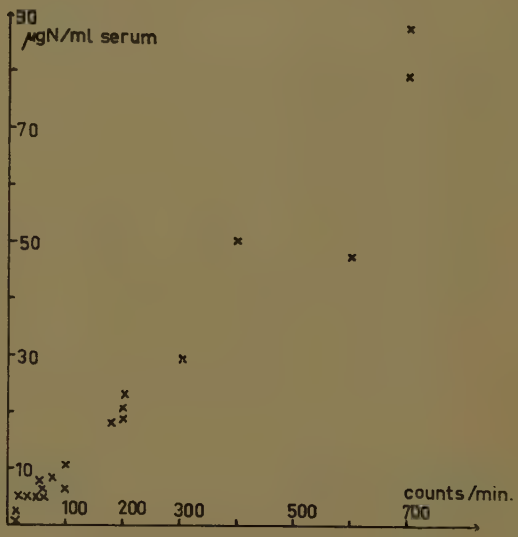
his reaction was used for the earlier cribed method for OCT determination. ulline-carbamyl-^{14}C was used as substrate the formation of radioactive CO_2 was easure of the OCT activity.

n order to avoid the use of the isotopic strate a method has now been worked in which the formation of NH_3 from ulline (reaction (1)) is measured.

METHOD

ne ml serum is incubated at 37° C for 24 hours one ml of a solution containing 0.2 m dl citrul- and 0.5 m sodium arsenate buffer pH 7.1. Per- ric acid (4-n, 0.2 ml) is added and the preci- e centrifuged off.

ne ml of the supernatant is transferred to the r chamber of a Conway microdiffusion stan- unit of plexiglas (outer diameter 65 mm). center chamber contains 1.3—2 ml 0.01 HCl. ee ml of potassium borate buffer (12.3 g HBO_3 ml 4-nKOH + aqua redest. ad 500 ml) are d in the outer chamber. After 2½ hours in-



Comparison of the results obtained for the OCT determination in serum by the citrulline-carbamyl-^{14}C method (abscissa in counts/min) and microdiffusion method (ordinate in µg N/ml serum). The OCT units in counts used here are only relative figures referring to applied standard conditions.

cubation at 37° C, the amount NH_3 in the centrum chamber is determined (Nesslerisation). Serum incubated with sodium arsenate buffer without citrulline and treated in the same way as the test serves as a blank. It is convenient to express the results of the OCT level in serum determined in this way as produced µgN/ml serum.

In order to prove that the results obtained by the microdiffusion technique correspond to the OCT levels in the samples 20 serum samples were analyzed with the citrulline-carbamyl-^{14}C method and the microdiffusion technique. For results see graph.

As the two methods seem to compare well, the quantitative determination of OCT in serum might be done without use of isotopic substrate.

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Medical Department,
Karolinska Sjukhuset and
Biochemical Department,
Karolinska Institutet,
Stockholm, Sweden.
July, 1957.

H. Reichard.

ıdinav. J. Clin. & Lab. Investigation 313—314, 9, 1957.

THE IN VITRO UPTAKE OF VITAMIN B_{12} BY DIPHYLLOBOTHRIUM LATUM AND ITS BLOCKAGE BY INTRINSIC FACTOR[1]

Recent results (Brante & Ernberg, 1957) tests on cases of pernicious tapeworm mia (PTA) strongly suggested that the petition between the host and Diphyllo-rium latum for vitamin B_{12} in the car-'s intestine, occasionally leading to PTA Bonsdorff, 1956), is primarily enacted be-en the intrinsic factor (IF) of the host the B_{12}-absorbing organ of the worm. a prerequisite the worm should be able idly to take up B_{12} in considerable ounts but more or less unable to absorb B_{12} complex. On the assumption that it ht maintain its *in vivo*-properties, freshly elled tapeworm was subjected to *in vitro*-eriments to test if the requirements are illed.

nalogous pieces of the middle portion of the m were immersed in a general medium com-d of (^{60}Co-)-B_{12} in normal saline (0.025 μC in 0.1 μg B_{12} per 45 cm worm and 2 ml of medium. pH about 6) with or without previous addition. After varying periods of incubation 7° the radioactivity of the worm pieces (after e washing) and of the medium (+ washings) measured. The test conditions which were ed in different directions and the findings will lescribed in more detail in a subsequent fuller rt.

s illustrated in Table I the worm is able efficiently to take up and bind in a e non-extractable state the B_{12} of an IF-free medium under the test conditions used. IF[1] blocks this uptake practically entirely. Further tests made clear that: the uptake power of the worm is restituted after removal of the IF; the concentration of incorporated B_{12} is at least as high in the lateral as in the medial part of the worm; the originally worm-bound B_{12} is again released to the surrounding fluid on autolysis; Taenia saginata takes up B_{12} much less efficiently than does the fish tapeworm.

Table I. *Uptake of vitamin B_{12} by tapeworm in and without the presence of intrinsic factor (IF).*

Addition to the general medium	Incubation time (hours)	Per cent of vitamin B_{12} of medium recovered in the worm
None	1	77.9
»	3.5	85.5
IF	1	< 0.6
»	3.5	< 0.8

It can be calculated from our results that 1 meter of fresh (equivalent to 1 g of dry) worm could incorporate some 0.2 μg B_{12} in 1 hour. If equally efficient *in vivo*, obviously the worm could seriously affect the results of tests of the absorption of peroral B_{12} (*e.g.* as in Nyberg's experiments (1956) and in the Schilling and Glass tests of our own studies (Brante & Ernberg, 1957), performed with 1 and 4.5 μg B_{12} respectively) as well as in time lead to B_{12} deprivation of the

[1] More precisely the blockage is due to B_{12}-ing principle, most of which in the IF-prepa-n used (Bendogen substance) appears to be ical with IF (Berlin, Berlin, Brante & Sjø-, to be published).

host, *i. e.* to PTA, carriers being known to harbour up to more than 100 meters of worm. And, if working as *in vitro,* any IF adequately available should be capable of reducing or preventing such effects of the worm (as in our Schilling tests 1957).

Thus our requirements for the existence of competition between worm and IF for B_{12}, set up in the introduction, seem to be fulfilled in our *in vitro* results. The question remains whether the latter are representative for the conditions *in vivo*. The state of the B_{12} incorporated *in vitro* — inextractable by saline and releasable during autolysis — compares to that of B_{12} taken up by the worm *in vivo* as reported by Nyberg (1956). The difference in power of B_{12} uptake between Diphyllobothrium latum and Taenia saginata *in vitro* reflects the difference established in their ability to cause B_{12} deprivation of the host. The last-mentioned two observations may be considered in favor of the behavior of the tapeworm being in principle similar *in vivo* as *in vitro* and we know nothing pointing in the opposite direction. Of course, the efficiency of the worm to take up B_{12} must vary with its surroundings. The influence of variation of the latter will be the subject of further studies as will the manner of entrance and binding of the B_{12} incorporated by the worm. For the present it seems probable that the B_{12} can enter into or through the intact worm main surface. The distribution found of the incorporated B_{12} over the lateral and medial parts of the worm may in some degree point in that direction. Whether B_{12} is taken up by simple exchang of, or as an actual addition to, the origin B_{12} content of the worm is not apparent fro our results. The B_{12} content of expelled wor is known to be about 2.3 μg/g dry matte wherefore exchange of some 0.2 μg/g d matter — the uptake in our experiments – is possible. It could, however, not adequate explain the power of the worm to cause with drawal of B_{12} from the host. Freshly expelle tapeworm may prove to be a suitable obje for studying these and other problems of B metabolism.

Central Laboratory and
Medical Department,
Centrallasarettet
Eskilstuna, Sweden
July 1957.

G. Bran
T. Ernber

SUMMARY

Expelled Diphyllobothrium latum wa found to take up very efficiently vitamin B from a fluid medium. Intrinsic factor[1] cou block this uptake. The findings support th authors' suggestion (1957) that the wor *in vivo* may compete with intrinsic factor f perorally ingested vitamin B_{12} and that that way, when successful, it may cause pern cious tapeworm anaemia.

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NEW METHOD FOR DETERMINING ALDOSACCHARIDES

or the past year we have studied the ling of para-amino-hippuric acid (PAH) para-amino benzoic acid (PAB) with monosaccharides. Aliquots of 0.4 M H and glucose are mixed and incubated 24 hours. About 50 per cent of the glucose en coupled with PAH giving a change of optical rotation and inhibiting diazotation ne aminogroup of the PAH-glucoside. On ng 10 volumes glacial acetic acid to such ixture and heating in a boiling waterbath ry dark black-brown color appears. We e tried to use an analogous method for rmination of sugar.

) Some compounds related to PAB have investigated and judged in three re-ts:

with water the reagents should give a extinction value.

with glucose a high extinction should lop.

the color should not have its absorption imum in the blue part of the spectrum.

esults in short:

AH: low blank; relatively low glucose r, maximum in blue.

AB: ditto.

rto-aminobenzoic acid (anthranilic acid): olor.

nilin: no color.

ara-phenylenediamine: high blank, rela-y low glucose color, maximum in blue.

ara-aminosalicylic acid (PAS): low k, relatively high glucose color, maxi-n at 520 mμ (see fig. 1).

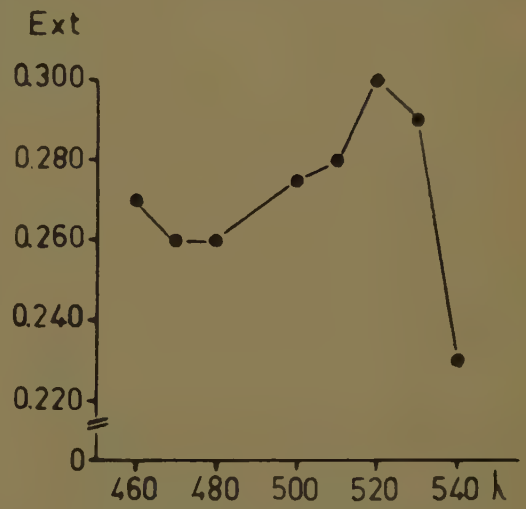

Fig. 1. Absorption spectrum of the "glucose color".

It seems to be of importance that there is an amino group in para position on a benzene ring against a carboxyl group. PAS seems to be the best substance tested.

B) Method for determination in water solutions.

Reagents:

1) glacial acetic acid (as free from aldehyde as possible. Aldehyde reaction according to Hopkins-Cole must not give more than a weak reaction).

2) Na-PAS 40 g + 100 g water. When kept at 4° C in the dark the solution is stable for at least some days.

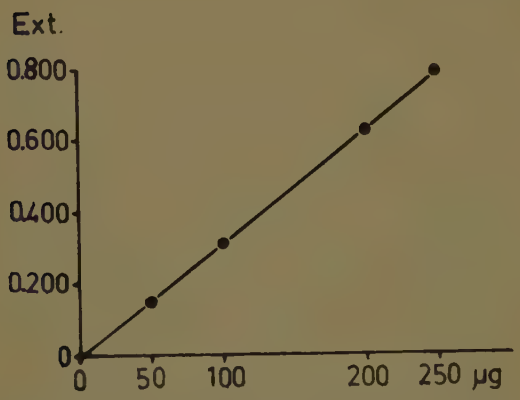

Fig. 2. Color read at 520 mμ with different amounts of sugar in water solution.

Procedure:

0.5 ml of the sample in water solution + 0.5 ml of reagent 2) + 5 ml reagent 1) are mixed in a long narow test-tube and warmed in boiling waterbath for 20 minutes. If precipitation occurs it is of no importance as it quickly dissolves. After 20 minutes the tubes are cooled to room temperature and the color is read at 520 mμ.

(Beckman B spectrophotometer is used. The cuvettes hold a thickness of 1 cm).

Results: The strength of the salmon pink color is within wide limits proportional to the sugar content (see fig. 2).

Ketosugars and acetone give no color comparable concentrations.

Acetaldehyde gives a brown color clear of a different tinge from the glucose colo At 520 mμ 45 mg per cent acetaldehyde giv the same extinction as 10 mg per cent gl couse. Galactose and mannose give the sa color and corresponding extinction.

The color of the sugars increases ve rapidly with boiling time from zero to minutes and after 20 minutes the increa continues very slowly. Sixteen hours af boiling the extinction values had increas up to 10 per cent but they were still on straight line passing through the origin.

C) Procedure for blood:

The following method has been used blood: 0.1 ml whole blood is mixed with ml 3 per cent trichloracetic acid. 0.5 ml supernatant is handled in the same way for the water solutions (see B).

Heparinized whole blood standing in roo temperature for one or two days gives ve low values corresponding to about 10 m per cent glucose.

On adding known amounts of glucose glycolized blood one gets values comparab with those of water solutions of glucose. W are working on the clinical standardizati and practical modifications of the method.

Central Laboratory
S:t Eriks Sjukhus
Stockholm, Sweden.
September 1957.

J. E
E. Hultma

BB

Bausch & Lombs kombinerede spectrofotometer og kolorimeter med indbygget monokromator. Bølgelængdeområdet 375 m μ—650 m μ, kan udvides til 950 m μ. Velegnet til såvel klinisk som industrielt brug.

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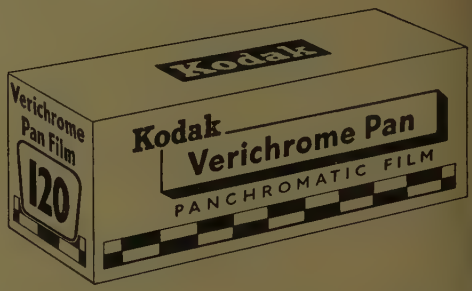

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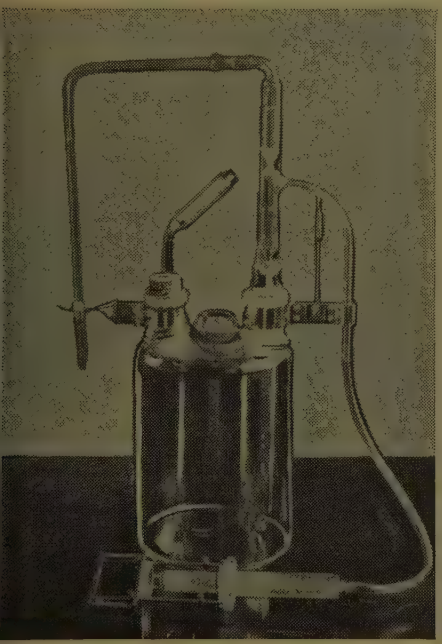

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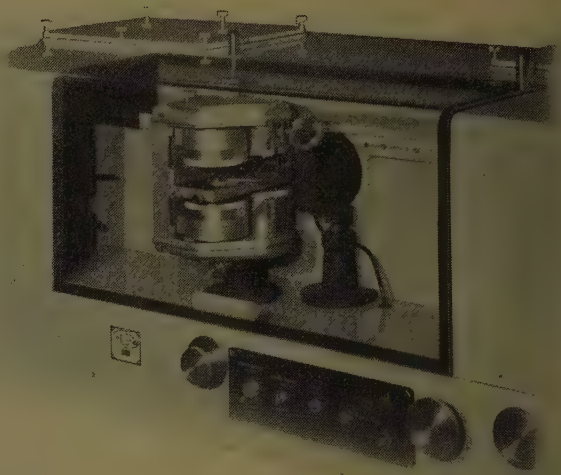

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